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(54) Title: NUCLEIC ACID LIGANDS AND METHODS FOR PRODUCING THE SAME

(57) Abstract

The present invention includes methods for the identification and production of improved nucleic acid ligands based on the SELEX process. Also included are nucleic acid ligands to the HIV-RT, HIV-1 Rev, HIV-1 tat, thrombin, and basic fibroblast growth factor proteins.

NUCLEIC ACID LIGANDS AND
METHODS FOR PRODUCING THE SAME

FIELD OF THE INVENTION

5   Described herein are methods for identifying and
producing nucleic acid ligands. Nucleic acid ligands
are double or single stranded DNA or RNA species that
bind specifically to a desired target molecule. The
basis for identifying nucleic acid ligands is a method
10   termed SELEX, an acronym for Systematic Evolution of
Ligands for EXponential enrichment.

          The methods of the present invention include means
for analyzing and applying the information learned from
the SELEX method to create an improved nucleic acid
15   ligand for the selected target. These methods include
computer modeling, boundary determination methods and
chemical modification methods. According to the
methods of this invention it is possible to determine:
1) which nucleic acid residues of a nucleic acid ligand
20   are critical in binding to the selected target; 2)
which nucleic acid residues affect the structural
conformation of the nucleic acid ligand; and 3) what is
the three-dimensional structure of the nucleic acid
ligand. This information allows for the identification
25   and production of improved nucleic acid ligands that
have superior binding capacity to the target as well as
enhanced structural stability. This information may
also be utilized to produce non-nucleic acid or hybrid-
nucleic acid species that also function as ligands to
30   the target. The methods of the present invention
further provide an analysis of the target species that
can be used in the preparation of therapeutic and/or
diagnostic methods.

          Specifically described herein are high-affinity
35   nucleic acid ligands to the HIV-RT, HIV-1 Rev, HIV-1
tat, thrombin, and basic fibroblast growth factor
(bFGF) proteins. Included within the scope of the
invention are modified nucleic acid ligands and mimetic
ligands that are informed by the nucleic acid ligands

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identified herein. Further included within the scope
of the invention are nucleic acid ligands capable of
modifying the biological activity of the target
molecule, for example, nucleic acid ligands that

5   inhibit the action of bFGF. Still further included in
the present invention are nucleic acid ligands
containing modified nucleotides.

BACKGROUND OF THE INVENTION

10   Most proteins or small molecules are not known to
specifically bind to nucleic acids. The known protein
exceptions are those regulatory proteins such as
repressors, polymerases, activators and the like which
function in a living cell to bring about the transfer

15   of genetic information encoded in the nucleic acids
into cellular structures and the replication of the
genetic material. Furthermore, small molecules such as
GTP bind to some intron RNAs.

            Living matter has evolved to limit the function of

20   nucleic acids to a largely informational role. The
Central Dogma, as postulated by Crick, both originally
and in expanded form, proposes that nucleic acids
(either RNA or DNA) can serve as templates for the
synthesis of other nucleic acids through replicative

25   processes that "read" the information in a template
nucleic acid and thus yield complementary nucleic
acids. All of the experimental paradigms for genetics
and gene expression depend on these properties of
nucleic acids: in essence, double-stranded nucleic

30   acids are informationally redundant because of the
chemical concept of base pairs and because replicative
processes are able to use that base pairing in a
relatively error-free manner.

            The individual components of proteins, the twenty

35   natural amino acids, possess sufficient chemical
differences and activities to provide an enormous
breadth of activities for both binding and catalysis.

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Nucleic acids, however, are thought to have narrower
chemical possibilities than proteins, but to have an
informational role that allows genetic information to
be passed from virus to virus, cell to cell, and
5   organism to organism. In this context nucleic acid
components, the nucleotides, must possess only pairs of
surfaces that allow informational redundancy within a
Watson-Crick base pair. Nucleic acid components need
not possess chemical differences and activities
10   sufficient for either a wide range of binding or
catalysis.

However, some nucleic acids found in nature do
participate in binding to certain target molecules and
even a few instances of catalysis have been reported.
15   The range of activities of this kind is narrow compared
to proteins and more specifically antibodies. For
example, where nucleic acids are known to bind to some
protein targets with high affinity and specificity, the
binding depends on the exact sequences of nucleotides
20   that comprise the DNA or RNA ligand. Thus, short
double-stranded DNA sequences are known to bind to
target proteins that repress or activate transcription
in both prokaryotes and eukaryotes. Other short
double-stranded DNA sequences are known to bind to
25   restriction endonucleases, protein targets that can be
selected with high affinity and specificity. Other
short DNA sequences serve as centromeres and telomeres
on chromosomes, presumably by creating ligands for the
binding of specific proteins that participate in
30   chromosome mechanics. Thus, double-stranded DNA has a
well-known capacity to bind within the nooks and
crannies of target proteins whose functions are
directed to DNA binding. Single-stranded DNA can also
bind to some proteins with high affinity and
35   specificity, although the number of examples is rather
smaller. From the known examples of double-stranded
DNA binding proteins, it has become possible to

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describe the binding interactions as involving various
protein motifs projecting amino acid side chains into
the major groove of B form double-stranded DNA,
providing the sequence inspection that allows
5   specificity.
        Double-stranded RNA occasionally serves as a
ligand for certain proteins, for example, the
endonuclease RNase III from E. coli. There are more
known instances of target proteins that bind to single-
10   stranded RNA ligands, although in these cases the
single-stranded RNA often forms a complex three-
dimensional shape that includes local regions of
intramolecular double-strandedness. The amino-acyl
tRNA synthetases bind tightly to tRNA molecules with
15   high specificity. A short region within the genomes of
RNA viruses binds tightly and with high specificity to
the viral coat proteins. A short sequence of RNA binds
to the bacteriophage T4-encoded DNA polymerase, again
with high affinity and specificity. Thus, it is
20   possible to find RNA and DNA ligands, either double- or
single-stranded, serving as binding partners for
specific protein targets. Most known DNA binding
proteins bind specifically to double-stranded DNA,
while most RNA binding proteins recognize single-
25   stranded RNA. This statistical bias in the literature
no doubt reflects the present biosphere's statistical
predisposition to use DNA as a double-stranded genome
and RNA as a single-stranded entity in the roles RNA
plays beyond serving as a genome. Chemically there is
30   no strong reason to dismiss single-stranded DNA as a
fully able partner for specific protein interactions.
        RNA and DNA have also been found to bind to
smaller target molecules. Double-stranded DNA binds to
various antibiotics, such as actinomycin D. A specific
35   single-stranded RNA binds to the antibiotic
thiostreptone; specific RNA sequences and structures
probably bind to certain other antibiotics, especially

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those whose functions is to inactivate ribosomes in a
target organism. A family of evolutionary related RNAs
binds with specificity and decent affinity to
nucleotides and nucleosides (Bass, B. and Cech, T.
5   (1984) Nature 308:820) as well as to one of the twenty
amino acids (Yarus, M. (1988) Science 240:1751).
Catalytic RNAs are now known as well, although these
molecules perform over a narrow range of chemical
possibilities, which are thus far related largely to
10   phosphodiester transfer reactions and hydrolysis of
nucleic acids.

          Despite these known instances, the great majority
of proteins and other cellular components are thought
not to bind to nucleic acids under physiological
15   conditions and such binding as may be observed is non-
specific. Either the capacity of nucleic acids to bind
other compounds is limited to the relatively few
instances enumerated supra, or the chemical repertoire
of the nucleic acids for specific binding is avoided
20   (selected against) in the structures that occur
naturally. The present invention is premised on the
inventors' fundamental insight that nucleic acids as
chemical compounds can form a virtually limitless array
of shapes, sizes and configurations, and are capable of
25   a far broader repertoire of binding and catalytic
functions than those displayed in biological systems.

          The chemical interactions have been explored in
cases of certain known instances of protein-nucleic
acid binding. For example, the size and sequence of
30   the RNA site of bacteriophage R17 coat protein binding
has been identified by Uhlenbeck and coworkers. The
minimal natural RNA binding site (21 bases long) for
the R17 coat protein was determined by subjecting
variable-sized labeled fragments of the mRNA to
35   nitrocellulose filter binding assays in which protein-
RNA fragment complexes remain bound to the filter
(Carey et al. (1983) Biochemistry 22:2601). A number

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of sequence variants of the minimal R17 coat protein
binding site were created in vitro in order to
determine the contributions of individual nucleic acids
to protein binding (Uhlenbeck et al. (1983) J. Biomol.
5   Structure Dynamics 1:539 and Romaniuk et al. (1987)
Biochemistry 26:1563). It was found that the
maintenance of the hairpin loop structure of the
binding site was essential for protein binding but, in
addition, that nucleotide substitutions at most of the
10   single-stranded residues in the binding site, including
a bulged nucleotide in the hairpin stem, significantly
affected binding. In similar studies, the binding of
bacteriophage Qβ coat protein to its translational
operator was examined (Witherell and Uhlenbeck (1989)
15   Biochemistry 28:71). The Qβ coat protein RNA binding
site was found to be similar to that of R17 in size,
and in predicted secondary structure, in that it
comprised about 20 bases with an 8 base pair hairpin
structure which included a bulged nucleotide and a 3
20   base loop. In contrast to the R17 coat protein binding
site, only one of the single-stranded residues of the
loop is essential for binding and the presence of the
bulged nucleotide is not required. The protein-RNA
binding interactions involved in translational
25   regulation display significant specificity.

Nucleic acids are known to form secondary and
tertiary structures in solution. The double-stranded
forms of DNA include the so-called B double-helical
form, Z-DNA and superhelical twists (Rich, A. et al.
30   (1984) Ann. Rev. Biochem. 53:791). Single-stranded RNA
forms localized regions of secondary structure such as
hairpin loops and pseudoknot structures (Schimmel, P.
(1989) Cell 58:9). However, little is known concerning
the effects of unpaired loop nucleotides on stability
35   of loop structure, kinetics of formation and
denaturation, thermodynamics, and almost nothing is
known of tertiary structures and three dimensional

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shape, nor of the kinetics and thermodynamics of
tertiary folding in nucleic acids (Tuerk et al. (1988)
Proc. Natl. Acad. Sci. USA 85:1364).

A type of in vitro evolution was reported in
replication of the RNA bacteriophage Qβ. Mills et al.
(1967) Proc. Natl. Acad. Sci USA 58:217; Levinsohn &
Spiegleman (1968) Proc. Natl. Acad. Sci. USA 60:866;
Levinsohn & Spiegelman (1969) Proc. Natl. Acad. Sci.
USA 63:805; Saffhill et al. (1970) J. Mol. Biol.
51:531; Kacian et al. (1972) Proc. Natl. Acad. Sci. USA
69:3038; Mills et al. (1973) Science 180:916. The
phage RNA serves as a poly-cistronic messenger RNA
directing translation of phage-specific proteins and
also as a template for its own replication catalyzed by
Qβ RNA replicase. This RNA replicase was shown to be
highly specific for its own RNA templates. During the
course of cycles of replication in vitro small variant
RNAs were isolated which were also replicated by Qβ
replicase. Minor alterations in the conditions under
which cycles of replication were performed were found
to result in the accumulation of different RNAs,
presumably because their replication was favored under
the altered conditions. In these experiments, the
selected RNA had to be bound efficiently by the
replicase to initiate replication and had to serve as a
kinetically favored template during elongation of RNA.
Kramer et al. (1974) J. Mol. Biol. 89:719 reported the
isolation of a mutant RNA template of Qβ replicase, the
replication of which was more resistant to inhibition
by ethidium bromide than the natural template. It was
suggested that this mutant was not present in the
initial RNA population but was generated by sequential
mutation during cycles of in vitro replication with Qβ
replicase. The only source of variation during
selection was the intrinsic error rate during
elongation by Qβ replicase. In these studies what was
termed "selection" occurred by preferential

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amplification of one or more of a limited number of
spontaneous variants of an initially homogenous RNA
sequence. There was no selection of a desired result,
only that which was intrinsic to the mode of action of
5   Qß replicase.

Joyce and Robertson (Joyce (1989) in RNA:
Catalysis, Splicing, Evolution, Belfort and Shub
(eds.), Elsevier, Amsterdam pp. 83-87; and Robertson
and Joyce (1990) Nature 344:467) reported a method for
10   identifying RNAs which specifically cleave single-
stranded DNA. The selection for catalytic activity was
based on the ability of the ribozyme to catalyze the
cleavage of a substrate ssRNA or DNA at a specific
position and transfer the 3'-end of the substrate to
15   the 3'-end of the ribozyme. The product of the desired
reaction was selected by using an oligodeoxynucleotide
primer which could bind only to the completed product
across the junction formed by the catalytic reaction
and allowed selective reverse transcription of the
20   ribozyme sequence. The selected catalytic sequences
were amplified by attachment of the promoter of T7 RNA
polymerase to the 3'-end of the cDNA, followed by
transcription to RNA. The method was employed to
identify from a small number of ribozyme variants the
25   variant that was most reactive for cleavage of a
selected substrate.

The prior art has not taught or suggested more
than a limited range of chemical functions for nucleic
acids in their interactions with other substances: as
30   targets for proteins evolved to bind certain specific
oligonucleotide sequences; and more recently, as
catalysts with a limited range of activities. Prior
"selection" experiments have been limited to a narrow
range of variants of a previously described function.
35   Now, for the first time, it will be understood that the
nucleic acids are capable of a vastly broad range of
functions and the methodology for realizing that

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capability is disclosed herein.

U.S. patent application serial number 07/536,428
filed June 11, 1990, of Gold and Tuerk, entitled
Systematic Evolution of Ligands by Exponential
5   Enrichment, and U.S. patent application serial no.
07/714,131 filed June 10, 1991 of Gold and Tuerk,
entitled Nucleic Acid Ligands (See also PCT/US91/04078)
describe a fundamentally novel method for making a
nucleic acid ligand for any desired target. Each of
10   these applications, collectively referred to herein as
the SELEX Patent Applications, is specifically
incorporated herein by reference.

The method of the SELEX Patent Applications is
based on the unique insight that nucleic acids have
15   sufficient capacity for forming a variety of two- and
three-dimensional structures and sufficient chemical
versatility available within their monomers to act as
ligands (form specific binding pairs) with virtually
any chemical compound, whether large or small in size.
20   The method involves selection from a mixture of
candidates and step-wise iterations of structural
improvement, using the same general selection theme, to
achieve virtually any desired criterion of binding
affinity and selectivity. Starting from a mixture of
25   nucleic acids, preferably comprising a segment of
randomized sequence, the method, termed SELEX herein,
includes steps of contacting the mixture with the
target under conditions favorable for binding,
partitioning unbound nucleic acids from those nucleic
30   acids which have bound to target molecules,
dissociating the nucleic acid-target pairs, amplifying
the nucleic acids dissociated from the nucleic acid-
target pairs to yield a ligand-enriched mixture of
nucleic acids, then reiterating the steps of binding,
35   partitioning, dissociating and amplifying through as
many cycles as desired.

While not bound by a theory of preparation, SELEX

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is based on the inventors' insight that within a
nucleic acid mixture containing a large number of
possible sequences and structures there is a wide range
of binding affinities for a given target. A nucleic

5   acid mixture comprising, for example a 20 nucleotide
randomized segment can have 4^{20} candidate
possibilities. Those which have the higher affinity
constants for the target are most likely to bind.
After partitioning, dissociation and amplification, a

10   second nucleic acid mixture is generated, enriched for
the higher binding affinity candidates. Additional
rounds of selection progressively favor the best
ligands until the resulting nucleic acid mixture is
predominantly composed of only one or a few sequences.

15   These can then be cloned, sequenced and individually
tested for binding affinity as pure ligands.

Cycles of selection and amplification are repeated
until a desired goal is achieved. In the most general
case, selection/amplification is continued until no

20   significant improvement in binding strength is achieved
on repetition of the cycle. The method may be used to
sample as many as about 10^{18} different nucleic acid
species. The nucleic acids of the test mixture
preferably include a randomized sequence portion as

25   well as conserved sequences necessary for efficient
amplification. Nucleic acid sequence variants can be
produced in a number of ways including synthesis of
randomized nucleic acid sequences and size selection
from randomly cleaved cellular nucleic acids. The

30   variable sequence portion may contain fully or
partially random sequence; it may also contain
subportions of conserved sequence incorporated with
randomized sequence. Sequence variation in test
nucleic acids can be introduced or increased by

35   mutagenesis before or during the selection/
amplification iterations.

In one embodiment of the method of the SELEX

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Patent Applications, the selection process is so
efficient at isolating those nucleic acid ligands that
bind most strongly to the selected target, that only
one cycle of selection and amplification is required.
5   Such an efficient selection may occur, for example, in
a chromatographic-type process wherein the ability of
nucleic acids to associate with targets bound on a
column operates in such a manner that the column is
sufficiently able to allow separation and isolation of
10   the highest affinity nucleic acid ligands.
        In many cases, it is not necessarily desirable to
perform the iterative steps of SELEX until a single
nucleic acid ligand is identified. The target-specific
nucleic acid ligand solution may include a family of
15   nucleic acid structures or motifs that have a number of
conserved sequences and a number of sequences which can
be substituted or added without significantly effecting
the affinity of the nucleic acid ligands to the target.
By terminating the SELEX process prior to completion,
20   it is possible to determine the sequence of a number of
members of the nucleic acid ligand solution family.
        A variety of nucleic acid primary, secondary and
tertiary structures are known to exist. The structures
or motifs that have been shown most commonly to be
25   involved in non-Watson-Crick type interactions are
referred to as hairpin loops, symmetric and asymmetric
bulges, psuedoknots and myriad combinations of the
same. Almost all known cases of such motifs suggest
that they can be formed in a nucleic acid sequence of
30   no more than 30 nucleotides. For this reason, it is
often preferred that SELEX procedures with contiguous
randomized segments be initiated with nucleic acid
sequences containing a randomized segment of between
about 20-50 nucleotides.
35   The SELEX Patent Applications also describe
methods for obtaining nucleic acid ligands that bind to
more than one site on the target molecule, and to

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nucleic acid ligands that include non-nucleic acid
species that bind to specific sites on the target. The
SELEX method provides means for isolating and
identifying nucleic acid ligands which bind to any
5   envisonable target. However, in preferred embodiments
the SELEX method is applied to situations where the
target is a protein, including both nucleic acid-
binding proteins and proteins not known to bind nucleic
acids as part of their biological function.
10   Little is known about RNA structure at high
resolution. The basic A-form helical structure of
double stranded RNA is known from fiber diffraction
studies. X-ray crystallography has yielded the
structure of a few tRNAs and a short poly-AU helix.
15   The X-ray structure of a tRNA/synthetase RNA/protein
complex has also been solved. The structures of two
tetranucleotide hairpin loops and one model pseudoknot
are know from NMR studies.
          There are several reasons behind the paucity of
20   structural data. Until the advent of _in vitro_ RNA
synthesis, it was difficult to isolate quantities of
RNA sufficient for structural work. Until the
discovery of catalytic RNAs, there were few RNA
molecules considered worthy of structural study. Good
25   tRNA crystals have been difficult to obtain,
discouraging other crystal studies. The technology for
NMR study of molecules of this size has only recently
become available.
          As described above, several examples of catalytic
30   RNA structures are known, and the SELEX technology has
been developed which selects RNAs that bind tightly to
a variety of target molecules - and may eventually be
able to select for new catalytic RNA structures as
well. It has become important to know the structure of
35   these molecules, in order to learn how exactly they
work, and to use this knowledge to improve upon them.
          It would be desirable to understand enough about

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RNA folding to be able to predict the structure of an
RNA with less effort than resorting to rigorous NMR,
and X-ray crystal structure determination. For both
proteins and RNAs, there has always been a desire to be
5   able to compute structures based on sequences, and with
limited (or no) experimental data.

         Protein structure prediction is notoriously
difficult. To a first approximation, the secondary
structure and tertiary structure of proteins form
10   cooperatively; protein folding can be approximated
thermodynamically by a two-state model, with completely
folded and completely unfolded states. This means that
the number of degrees of freedom for modeling a protein
structure are very large; without predictable
15   intermediates, one cannot break the prediction problem
into smaller, manageable sub problems. In contrast,
RNAs often appear to make well-defined secondary
structures which provide more stability than the
tertiary interactions. For example, the tertiary
20   structure of tRNA can be disrupted without disrupting
the secondary structure by chelation of magnesium or by
raising the temperature. Secondary structure
prediction for RNAs is well-understood, and is
generally quite accurate for small RNA molecules. For
25   RNAs, structural prediction can be broken into
subproblems; first, predict the secondary structure;
then, predict how the resulting helices and remaining
single strands are arranged relative to each other.

         For RNA, the first attempts at structural
30   prediction were for tRNAs. The secondary structure of
the canonical tRNA cloverleaf was known from
comparative sequence analysis, reducing the problem to
one of arranging four short A-form helices in space
relative to each other. Manual CPK modeling, back-of-
35   the-envelope energy minimization, and a few distance
restraints available from crosslinking studies and
phylogenetic covariations were used to generate a tRNA

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model - which unfortunately proved wrong when the first
crystal structure of phenylalanine tRNA was solved a
few years later.

Computer modeling has supplanted manual modeling,
relieving the model-builder of the difficulties imposed
by gravitation and mass. Computer modeling can only be
used without additional experimental data for instances
in which a homologous structure is known; for instance,
the structure of the 3' end of the turnip yellow mosaic
virus RNA genome was modeled, based on the known 3D
structure of tRNA and the knowledge that the 3' end of
TYMV is recognized as tRNA-like by a number of cellular
tRNA modification enzymes. This model was the first 3D
model of an RNA pseudoknot; the basic structure of an
isolated model pseudoknot has been corroborated by NMR
data.

Computer modeling protocols have been used,
restrained by the manual inspection of chemical and
enzymatic protection data, to model the structures of
several RNA molecules. In one isolated substructure,
one model for the conformation of a GNRA
tetranucleotide loop has been shown to be essentially
correct by NMR study of an isolated GNRA hairpin loop.

Francois Michel ((1989) Nature 342:391) has
constructed a model for the catalytic core of group I
introns. Like the tRNAs, the secondary structure of
group I intron cores is well-known from comparative
sequence analysis, so the problem is reduced to one of
properly arranging helices and the remaining single-
stranded regions. Michel (1989) supra, analyzed an
aligned set of 87 group I intron sequences by eye and
detected seven strong pairwise and triplet covariations
outside of the secondary structure, which he
interpreted as tertiary contacts and manually
incorporated as restraints on his model. As yet, there
is no independent confirmation of the Michel model.

Others have attempted to devise an automated

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procedure to deal with distance restraints from crosslinking, fluorescence transfer, or phylogentic co-variation. The RNA is treated as an assemblage of cylinders (A-form helices) and beads (single-stranded residues), and a mathematical technique called distance geometry is used to generate arrangements of these elements which are consistent with a set of distance restraints. Using a small set of seven distance restraints on the phenylalanine tRNA tertiary structure, this protocol generated the familiar L-form of the tRNA structure about 2/3 of the time.

The HIV-1 _tat_ protein activates transcription in the long terminal repeat (LTR) of the viral genome of HIV-1. See, Cullen et al. (1989) Cell 58:423. The mechanism of activation is unclear or at least controversial, but requires that the transcribed RNA contain a specific hairpin structure with a trinucleotide bulge (called TAR). The natural TAR RNA and the site of _tat_ interaction is shown in Figure 25. A small basic domain of the _tat_ protein has been shown to interact directly with the TAR RNA sequence. See, Weeks et al. (1990) Science 249:1281; Roy et al. (1990) Genes Dev. 4:1365; Calnan et al. (1991a) Genes Dev. 5:201. Arginines within this basic domain are apparently crucial to the interaction. See, Calnan et al. (1991a) _supra_; Subramanian et al. (1991) EMBO 10:2311-2318; Calnan et al (1991b) Science 252:1167-1171. Arginine alone is specifically bound by the TAR RNA sequence and may compete for _tat_ protein binding. See, Tao et al. (1992) Proc. Natl. Acad. Sci. USA 89:2723-2726; Puglisi et al. (1992) Science 257:76-80.

Tat - TAR interactions alone are insufficient to support transactivation; presumably a cellular factor - - a 68 kD loop binding protein -- is required for cooperative binding with the _tat_ protein to TAR, and subsequent _in vivo_ or _in vitro_ transactivation. See, Marciniak et al. (1990a) Proc. Natl. Acad. Sci. USA

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87:3624; Marciniak et al. (1990b) Cell 63:791.
Overexpression of the TAR sequence in retrovirally
transformed cell lines renders them highly resistant to
HIV-1 infections. See, Sullenger et al. (1990) Cell
5   63:601.

Thrombin is a multifunctional serine protease that
has important procoagulant and anticoagulant
activities. As a procoagulant enzyme thrombin clots
fibrinogen, activates clotting factors V, VIII, and
10   XIII, and activates platelets. The specific cleavage
of fibrinogen by thrombin initiates the polymerization
of fibrin monomers, a primary event in blood clot
formation. The central event in the formation of
platelet thrombi is the activation of platelets from
15   the "nonbinding" to the "binding" mode and thrombin is
the most potent physiologic activator of platelet
aggregation (Berndt and Phillips (1981) in Platelets in
Biology and Pathology, J.L. Gordon, ed. (Amsterdam:
Elsevier/North Holland Biomedical Press), pp. 43-74;
20   Hansen and Harker (1988) Proc. Natl. Acad. Sci. USA
85:3184; Eidt et al. (1989) J. Clin. Invest. 84:18).
Thus, as a procoagulant, thrombin plays a key role in
the arrest of bleeding (physiologic hemostasis) and
formation of vasoocclusive thrombi (pathologic
25   thrombosis).

As an anticoagulant thrombin binds to
thrombomodulin (TM), a glycoprotein expressed on the
surface of vascular endothelial cells. TM alters
substrate specificity from fibrinogen and platelets to
30   protein C through a combination of an allosteric change
in the active site conformation and an overlap of the
TM and fibrinogen binding sites on thrombin. Activated
protein C, in the presence of a phospholipid surface,
Ca^{2+}, and a second vitamin K-dependent protein
35   cofactor, protein S, inhibits coagulation by
proteolytically degrading factors Va and VIIIa. Thus
the formation of the thrombin-TM complex converts

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thrombin from a procoagulant to an anticoagulant
enzyme, and the normal balance between these opposing
activities is critical to the regulation of hemostasis.

Thrombin is also involved in biological responses
that are far removed from the clotting system (reviewed
in Shuman (1986) Ann. N. Y. Acad. Sci. 485:349; Marx
(1992) Science 256:1278). Thrombin is chemotactic for
monocytes (Bar-Shavit et al. (1983) Science 220:728),
mitogenic for lymphocytes (Chen et al. (1976) Exp. Cell
Res. 101:41), mesenchymal cells (Chen and Buchanan
(1975) Proc. Natl. Acad. Sci. USA 72:131), and
fibroblasts (Marx (1992) supra). Thrombin activates
endothelial cells to express the neutrophil adhesive
protein GMP-140 (PADGEM) (Hattori et al. (1989) J.
Biol. Chem. 264:7768) and produce platelet-derived
growth factor (Daniel et al. (1986) J. Biol. CHem.
261:9579). Recently it has been shown that thrombin
causes cultured nerve cells to retract their neurites
(reviewed in Marx (1992) supra.

The mechanism by which thrombin activates
platelets and endothelial cells is through a functional
thrombin receptor found on these cells. A putative
thrombin cleavage site (LDR/S) in the receptor suggests
that the thrombin receptor is activated by proteolytic
cleavage of the receptor. This cleavage event
"unmasks" an N-terminal domain which then acts as the
ligand, activating the receptor (Vu et al. (1991) Cell
64:1054).

Vascular injury and thrombus formation represent
the key events in the pathogenesis of various vascular
diseases, including atherosclerosis. The pathogenic
processes of the activation of platelets and/or the
clotting system leading to thrombosis in various
disease states and in various sites, such as the
coronary arteries, cardiac chambers, and prosthetic
heart valves, appear to be different. Therefore, the
use of a platelet inhibitor, an anticoagulant, or a

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combination of both may be required in conjunction with thrombolytics to open closed vessels and prevent reocclusion.

Controlled proteolysis by compounds of the
5   coagulation cascade is critical for hemostasis. As a result, a variety of complex regulatory systems exist that are based, in part, on a series of highly specific protease inhibitors. In a pathological situation functional inhibitory activity can be interrupted by
10   excessive production of active protease or inactivation of inhibitory activity. Perpetuation of inflammation in response to multiple trauma (tissue damage) or infection (sepsis) depends on proteolytic enzymes, both of plasma cascade systems, including thrombin, and
15   lysosomal origin. Multiple organ failure (MOF) in these cases is enhanced by the concurrently arising imbalance between proteases and their inhibitory regulators. An imbalance of thrombin activity in the brain may lead to neurodegenerative diseases.
20   Thrombin is naturally inhibited in hemostasis by binding to antithrombin III (ATIII), in a heparin-dependent reaction. Heparin exerts its effect through its ability to accelerate the action of ATIII. In the brain, protease nexin (PN-1) may be the natural
25   inhibitor of thrombin to regulate neurite outgrowth.

Heparin is a glycosoaminoglycan composed of chains of alternating residues of D-glucosamine and uronic acid. Heparin is currently used extensively as an anticoagulant in the treatment of unstable angina,
30   pulmonary embolism, atherosclerosis, thrombosis, and following myocardial infarction. Its anticoagulant effect is mediated through its interaction with ATIII. When heparin binds ATIII, the conformation of ATIII is altered, and it becomes a significantly enhanced
35   inhibitor of thrombin. Although heparin is generally considered to be effective for certain indications, it is believed that the physical size of the ATIII•heparin

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complex prevents access to much of the biologically
active thrombin in the body, thus diminishing its
ability to inhibit clot formation. Side effects of
heparin include bleeding, thrombocytopenia,
osteoporosis, skin necrosis, alpe, hypersensitivity and
hypoaldoseronism.

Hirudin is a potent peptide inhibitor of thrombin
derived from the European medicinal leech <u>Hirudis</u>
<u>medicinalis</u>. Hirudin inhibits all known functions of
α-thrombin, and has been shown to bind thrombin at two
separate sites kinetically; a high affinity site at or
near the catalytic site for serine protease activity
and a second anionic exosite. The anionic exosite also
binds fibrinogen, heparin, TM and probably the receptor
involved in mediating the activation of platelets and
endothelial cells. A C-terminal hirudin peptide --
which has been shown by co-crystallization with
thrombin to bind in the anionic exosite -- has
inhibitory effects on fibrin formation, platelet and
endothelial cell activation, and Protein C activation
via TM binding, presumably by competing for binding at
this site. This peptide does not inhibit proteolytic
activity towards tripeptide chromogenic substrates,
Factor V or X.

The structure of thrombin makes it a particularly
desirable target for nucleic acid binding, due to the
anionic exosite. Site-directed mutagenesis within this
site has shown that fibrinogen-clotting and TM binding
activities are separable. Conceivably, an RNA ligand
could be selected that has procoagulatory and/or
anticoagulatory effects depending on how it interacts
with thrombin, i.e., which substrate it mimics.

A single stranded DNA ligand to thrombin has been
prepared according to a procedure identical to SELEX.
<u>See</u>, Bock et al. (1992) Nature <u>355</u>:564. A consensus
ligand was identified after relatively few rounds of
SELEX were performed, that was shown to have some

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ability to prevent clot formation _in vitro_. The ligand
is the 15mer DNA 5'GGTTGGTGTGGTTGG-3', referred to
herein as G15D (SEQ ID NO:1). The symmetrical nature
of the primary sequence suggests that G15D has a
regular fixed tertiary structure. The kD of G15D to
thrombin is about 2 x 10⁻⁷. For effective thrombin
inhibition as an anticoagulant, the stronger the
affinity of the ligand to thrombin the better.

Basic fibroblast growth factor (bFGF) is a
multifunctional effector for many cells of mesenchymal
and neuroectodermal origin (Rifkin & Moscatelli (1989)
J. Cell Biol. 109:1; Baird & Bohlen (1991) in Peptide
Growth Factors and Their Receptors (Sporn, M. B. &
Roberts, A. B., eds.); pp. 369-418, Springer, N.Y.;
Basilico & Moscatelli (1992) Adv. Cancer Res. 59:115).
It is one of the most studied and best characterized
members of a family of related proteins that also
includes acidic FGF (Jaye et al. (1986) Science
233:541; Abraham et al. (1986) Science 233:545), int-2
(Moore et al. (1986) EMBO J. 5:919), kFGF/hst/KS3
(Delli-Bovi et al. (1987) Cell 50:729; Taira et al.
(1987) Proc. Natl. Acad. Sci. USA 84:2980), FGF-5 (Zhan
et al. (1988) Mol. Cell. Biol. 8:3487), FGF-6 (Marics
et al. (1989) Oncogene 4:335) and keratinocyte growth
factor/FGF-7 (Finch et al. (1989) Science 245:752).

In vitro, bFGF stimulates cell proliferation,
migration and induction of plasminogen activator and
collagenase activities (Presta et al. (1986) Mol. Cell.
Biol. 6:4060; Moscatelli et al. (1986) Proc. Natl.
Acad. Sci. USA 83:2091; Mignatti et al. (1989) J. Cell
Biol. 108:671). _In vivo_, it is one of the most potent
inducers of neovascularization. Its angiogenic
activity _in vivo_ suggests a role in tissue remodeling
and wound healing but also in some disease states that
are characterized by pathological neovascularization
such as tumor proliferation, tumor metastasis, diabetic
retinopathy and rheumatoid arthritis (Folkman &

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Klagsbrun (1987) Science 235:442; Gospodarowitz (1991)
Cell Biology Reviews 25:307).

      Although bFGF does not have a signal sequence for
secretion, it is found on both sides of the plasma
5   membrane, presumably being exported via exocytosis
(Vlodavsky et al. (1991) Trends Biol. Sci. 16:268;
Mignatti & Rifkin (1991) J. Cell. Biochem. 47:201).
In the extracellular matrix, it is typically associated
with a fraction that contains heparan sulfate
10   proteoglycans. Indeed, heparin affinity chromatography
has been a useful method for purification of this and
other heparin-binding growth factors. In cell culture,
bFGF binds to low- and high-affinity sites. The low-
affinity sites are composed of cell-associated heparan
15   sulfate proteoglycans to which bFGF binds with
approximately nanomolar affinity (Moscatelli (1987) J.
Cell. Physiol. 131:123). All biological effects of
bFGF are mediated through interaction with the high-
affinity binding sites (10-100 pM) that represent the
20   dimeric tyrosine kinase FGF receptor (Ueno et al.
(1992) J. Biol. Chem. 267:1470). Five FGF receptor
genes have been identified to date, each of which can
produce several structural variants as a result of
alternative mRNA splicing (Armstrong et al. (1992)
25   Cancer Res. 52:2004; Ueno et al. (1992) supra). There
is by now substantial evidence that the low- and the
high-affinity binding sites act cooperatively in
determining the overall affinity of bFGF. Experiments
with mutant cell lines that are deficient in
30   glycosaminoglycan synthesis (Yayon et al. (1991) Cell
64:841) or heparitinase treated cells (Rapraeger et al.
(1991) Science 252:1705) have shown that binding of
either cell-associated heparan sulfate or, in its
absence, exogenously added heparin to bFGF is required
35   for signaling via the tyrosine kinase receptor. Recent
resolution of observed Kd into its kinetic components
demonstrates that while the association rates of bFGF

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to the low- and the high-affinity sites are comparable, the _dissociation_ rate of bFGF from the cell surface receptor is 23-fold slower than that for the cell-associated heparan sulfate (Nugent & Edelman (1992)

5   Biochemistry 31:8876). The slower off-rate, however, is only observed when the receptor is bound to the cell surface suggesting that simultaneous binding to both sites contributes to the overall high-affinity binding. This is plausible in light of the observation that the

10   heparin-binding and the receptor-binding sites are located on adjacent but separate regions of the molecule, as determined from the recently solved X-ray crystal structure of bFGF (Zhang et al. (1991) Proc. Natl. Acad. Sci. USA 88:3446; Eriksson et al. (1991)

15   Proc. Natl. Acad. Sci. USA 88:3441; Ago et al. (1991) J. Biochem. 110:360; Zhu et al. (1991) Science 251:90).

       The idea that bFGF antagonists may have useful medicinal applications is not new (reviewed in Gospodarowicz (1991) _supra_). bFGF is now known to play

20   a key role in the development of smooth-muscle cell lesions following vascular injury (Reidy et al. Circulation, Suppl. III 86:III-43). Overexpression of bFGF (and other members of the FGF family) is correlated with many malignant disorders (Halaban et

25   al. (1991) Ann. N. Y. Acad. Sci. 638:232; Takahashi et al. (1990) Proc. Natl. Acad. Sci. USA 87:5710; Fujimoto et al. (1991) Biochem. Biophys. Res. Commun. 180:386) and recently, neutralizing anti-bFGF antibodies have been found to suppress solid tumor growth _in vivo_ by

30   inhibiting tumor-linked angiogenesis (Hori et al. (1991) Cancer Res. 51:6180). Notable in this regard is the recent therapeutic examination of suramin, a polysulfated naphthalene derivative with known antiprotozoal activity, as an anti-tumor agent.

35   Suramin is believed to inhibit the activity of bFGF through binding in the polyanion binding site and disrupting interaction of the growth factor with its

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receptor (Middaugh et al. (1992) Biochemistry 31:9016; Eriksson et al. (1992) supra). In addition to having a number of undesirable side effects and substantial toxicity, suramin is known to interact with several other heparin-binding growth factors which makes linking of its beneficial therapeutic effects to specific drug-protein interactions difficult (La Rocca et al. (1990) Cancer Cells 2:106). Anti-angiogenic properties of certain heparin preparations have also been observed (Folkman et al. (1983) Science 221:719; Crum et al. (1985) Science 250:1375) and these effects are probably based at least in part on their ability to interfere with bFGF signaling. While the specific heparin fraction that contributes to bFGF binding is now partially elucidated (Ishai-Michaeli et al. (1992) Biochemistry 31:2080; Turnbull et al. (1992) J. Biol. Chem. 267:10337), a typical heparin preparation is heterogeneous with respect to size, degree of sulfation and iduronic acid content. Additionally, heparin also affects many enzymes and growth factors. Excluding monoclonal antibodies, therefore, specific antagonists of bFGF are not known.

SUMMARY OF THE INVENTION

The present invention includes methods for identifying and producing nucleic acid ligands and the nucleic acid ligands so identified and produced. The SELEX method described above allows for the identification of a single nucleic acid ligand or a family of nucleic acid ligands to a given target. The methods of the present invention allow for the analysis of the nucleic acid ligand or family of nucleic acid ligands obtained by SELEX in order to identify and produce improved nucleic acid ligands.

Included in this invention are methods for determining the three-dimensional structure of nucleic acid ligands. Such methods include mathematical

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modeling and structure modifications of the SELEX
derived ligands. Further included are methods for
determining which nucleic acid residues in a nucleic
acid ligand are necessary for maintaining the three-
dimensional structure of the ligand, and which residues
interact with the target to facilitate the formation of
ligand-target binding pairs.

In one embodiment of the present invention,
nucleic acid ligands are desired for their ability to
inhibit one or more of the biological activities of the
target. In such cases, methods are provided for
determining whether the nucleic acid ligand effectively
inhibits the desired biological activity.

Further included in this invention are methods for
identifying tighter-binding RNA ligands and smaller,
more stable ligands for use in pharmaceutical or
diagnostic purposes.

The present invention includes improved nucleic
acid ligands to the HIV-RT and HIV-1 Rev proteins.
Also included are nucleic acid sequences that are
substantially homologous to and that have substantially
the same ability to bind HIV-RT or the HIV-1 Rev
protein as the nucleic acid ligands specifically
identified herein.

Also included within the scope of the invention is
a method for performing sequential SELEX experiments in
order to identify extended nucleic acid ligands. In
particular, extended nucleic acid ligands to the HIV-RT
protein are disclosed. Nucleic acid sequences that are
substantially homologous to and that have substantially
the same ability to bind HIV-RT as the extended HIV-RT
nucleic acid ligands are also included in this
invention.

Included within the scope of the invention are
nucleic acid ligands to the HIV-1 tat protein. More
specifically, RNA sequences have been identified that
are capable of binding to the tat protein. Included

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within the invention are the nucleic acid ligand
solutions shown in Figures 26 and 27.

Further included in this invention is a method of
identifying nucleic acid ligands and ligand solutions
5   to the HIV-1 _tat_ protein comprising the. steps of a)
preparing a candidate mixture of nucleic acids; b)
partitioning between members of said candidate mixture
on the basis of affinity to the _tat_ protein; and c)
amplifying the selected molecules to yield a mixture of
10   nucleic acids enriched for nucleic acid sequences with
a relatively higher affinity for binding to the _tat_
protein.

Further included in this invention are nucleic
acid ligands to thrombin. More specifically, RNA
15   sequences have been identified that are capable of
binding to thrombin. Included within the invention are
the nucleic acid ligand solutions shown in Figures 29
and 30.

Further included in this invention is a method of
20   identifying nucleic acid ligands and ligand solutions
to thrombin comprising the steps of a) preparing a
candidate mixture of nucleic acids; b) partitioning
between members of said candidate mixture on the basis
of affinity to thrombin; and c) amplifying the selected
25   molecules to yield a mixture of nucleic acids enriched
for nucleic acid sequences with a relatively higher
affinity for binding to thrombin.

More specifically, the present invention includes
the RNA ligands to thrombin identified according to the
30   above-described method, including those ligands listed
in Figure 29 (SEQ ID NO:137-155). Also included are
RNA ligands to thrombin that are substantially
homologous to any of the given ligands and that have
substantially the same ability to bind to thrombin.
35   Further included in this invention are RNA ligands to
thrombin that have substantially the same structural
form as the ligands presented herein and that have

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substantially the same ability to bind thrombin.

Further included in this invention are nucleic
acid ligands to bFGF. Specifically, RNA sequences are
provided that are capable of binding specifically to
bFGF. Included within the invention are the nucleic
acid ligand sequences shown in Tables II-IV (SEQ ID
NO:27-89).

Also included in this invention are nucleic acid
ligands of bFGF that are inhibitors of bFGF.
Specifically, RNA ligands are identified and described
which inhibit the binding of bFGF to its receptors.

Further included in this invention is a method of
identifying nucleic acid ligands and ligand sequences
to bFGF comprising the steps of a) preparing a
candidate mixture of nucleic acids; b) partitioning
between members of said candidate mixture on the basis
of affinity to bFGF; and c) amplifying the selected
molecules to yield a mixture of nucleic acids enriched
for nucleic acid sequences with a relatively higher
affinity for binding to bFGF.

More specifically, the present invention includes
the RNA ligands to bFGF identified according to the
above-described method, including those ligands listed
in Tables II-IV. Also included are RNA ligands to bFGF
that are substantially homologous to any of the given
ligands and that have substantially the same ability to
bind and inhibit bFGF. Further included in this
invention are RNA ligands to bFGF that have
substantially the same structural form as the ligands
presented herein and that have substantially the same
ability to bind and inhibit bFGF.

The present invention also includes modified
nucleotide sequences based on the nucleic acid ligand
sequences identified herein and mixtures of the same.
Specifically included in this invention are RNA
ligands, that have been modified at the ribose and/or
phosphate and/or base positions to increase <u>in vivo</u>

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stability of the RNA ligand. Other modifications to
RNA ligands are encompassed by this invention,
including specific alterations in base sequence, and
additions of nucleic acids or non-nucleic acid moieties
5   to the original compound.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the consensus pseudoknot derived
from primary and secondary SELEX experiments describing
10   high affinity inhibitory ligands of HIV-1 reverse
transcriptase (HIV-RT). The consensus secondary
structure is a pseudoknot; the 5' helix of that
pseudoknot (Stem 1) is conserved at the primary
sequence level and the 3' helix or Stem 2 is not. X
15   indicates a nucleotide position that is non-conserved;
X-X' indicates a preferred base-pair. The 26
nucleotide positions are numbered as shown.

Figure 2 depicts refinement of the 5' information
20   boundary. A set of model ligands were synthesized with
T7 RNA polymerase from template oligos. Milligan et al.
(1987) Nucl. Acid. Res. 15:8783). Illustrated in the
upper left is the complete ligand B. On the right
margin are shown the variations in the individual
25   ligands A through E that occur in the boxed areas. In
the graph are shown the individual binding curves for
these model ligands.

Figure 3 depicts the effect of various nucleotide
30   substitutions within the ligand B sequence on binding
to HIV-RT. Illustrated are the various substitutions
and resultant affinities to HIV-RT expressed relative
to the binding of ligand B. Ligand B was a control
tested in each experiment; the affinity of ligand B is
35   normalized as 1.0 and the relative affinity (Kd of
ligand B is divided by the Kd of each ligand) is shown.
Also shown are the affinities of various truncations of

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ligand B. The value associated with the asterisked G-G
which replaces U1-G16 comes from ligand C of Figure 2.

        Figure 4 depicts a chemical probe of the native
5   versus denatured conformations of ligand B. The
     various nucleotides of ligand B were reacted with
     chemicals under native and denaturing conditions,
     assayed for the modified positions, electrophoresed and
     visualized for comparison. ▮ indicate highly reactive
10   base-pairing groups of the base at that position and ▯
     partially reactivity; ▲ indicates strong reactivity of
     purine N7 positions and ▵ partial reactivity (to
     modification with DEPC). The question marks indicate
     that these positions on G(-2) and G(-1) could not be
15   distinguished due to band crowding on the gel.

        Figure 5 depicts reactivities of modifiable groups
     of ligand B when bound to HIV-RT. Diagrammed are those
     groups that show altered reactivity when bound to
20   HIV-RT as compared to that of the native conformation.

        Figure 6 depicts modification interference results
     for ligand B complexing with HIV-RT. Symbols for
     modification are as in the boxed legend. The
25   modifications indicated are those that are strongly
     (filled symbols) or partially (unfilled symbols)
     selected against by binding to HIV-RT (reflected by
     decreased modification at those positions in the
     selected population).
30
        Figure 7 depicts substitution of 2'-methoxy for
     hydroxyl on the riboses of the ligand B sequence shown
     in the upper right. Open circles represent hydroxyl
     groups at indicated positions and filled circles
35   indicated methoxy substitution.

        Figure 8 depicts selection by HIV-RT from mixed

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populations of 2'-methoxy ribose versus 2'-hydroxyl at
positions U1 through A5 and A12 through A20. An
oligonucleotide was synthesized with the following
sequence (SEQ ID NO:2):

5

5'-(AAAAA)$_d$(UCCGA)$_x$(AGUGCA)$_m$(ACGGGAAAA)$_x$(UGCACU)$_{m-3}$,

where subscripted "d" indicates 2'-deoxy, subscripted
"x" that those nucleotides are mixed 50-50 for
10   phosphoramidite reagents resulting in 2'-methoxy or
2'-hydroxyl on the ribose, and subscripted "m"
indicating that those nucleotides are all 2'-methoxy on
the ribose.

15   Figure 9 shows the starting RNA and the collection
of sequences (SEQ ID NO:115-135) obtained from SELEX
with HIV-RT as part of a walking experiment.

Figure 10 illustrates the consensus extended
20   HIV-RT ligand (SEQ ID NO:136) obtained from the list of
sequences shown in Figure 9.

Figure 11 illustrates the revised description of
the pseudoknot ligand of HIV-RT. In addition to the
25   labeling conventions of Figure 1, the S-S' indicates
the preferred C-G or G-C base-pair at this position.

Figure 12 shows the sequence of a high-affinity
RNA ligand for HIV-1 Rev protein obtained from SELEX
30   experiments. Shown is the numbering scheme used for
reference to particular bases in the RNA. This
sequence was used for chemical modification with ENU.
Figure 12 also shows at a) the extended RNA sequence
used in chemical modification experiments with DMS,
35   kethoxal, CMCT, and DEPC. The sequence of the
oligonucleotide used for primer extension of the
extended ligand sequence is shown at b).

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Figure 13 depicts the results of chemical
modification of the HIV-1 Rev ligand RNA under native
conditions. a) lists chemical modifying agents, their
specificity, and the symbols denoting partial and full
5   modification. The RNA sequence is shown, with degree
and type of modification displayed for every modified
base. b) depicts the helical, bulge, and hairpin
structural elements of the HIV-1 Rev RNA ligand
corresponding to the modification and computer
10   structural prediction data.

Figure 14 depicts the results of chemical
modification of the ligand RNA that interferes with
binding to the HIV-1 Rev protein. Listed are the
15   modifications which interfere with protein binding,
classified into categories of strong interference and
slight interference. Symbols denote either base-
pairing modifications, N7 modifications, or phosphate
modifications.
20

Figure 15 depicts the modification interference
values for phosphate alkylation. Data is normalized to
A17 3' phosphate.

25   Figure 16 depicts the modification interference
values for DMS modification of N3C and N1A. Data is
normalized to C36; A34.

Figure 17 depicts the modification intereference
30   values for kethoxal modification of N1G and N2G. Data
is normalized to G5.

Figure 18 depicts the modification interference
values for CMCT modification of N3U and N1G. Data is
35   normalized to U38.

Figure 19 depicts the modification interference

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values for DEPC modification of N7A and N7G. Data
normalied to G19; A34.

Figure 20 depicts the chemical modification of the
RNA ligand in the presence of the HIV-1 Rev protein.
Indicated are those positions that showed either
reduced modification or enhanced modification in the
presence of protein as compared to modification under
native conditions but without protein present.

Figure 21 shows the 5' and 3' sequences which
flank the "6a" biased random region used in SELEX. The
template which produced the initial RNA population was
constructed from the following oligonucleotides:

5'-CCCGGATCCTCTTTACCTCTGTGTGagatacagagtccacaaacgtgttc
tcaatgcacccGGTCGGAAGGCCATCAATAGTCCC-3' (template
oligo) (SEQ ID NO:3)

5'-CCGAAGCTTAATACGACTCACTATAGGGACTATTGATGGCCTTCCGACC-3'
(5' primer) (SEQ ID NO:4)

5'-CCCGGATCCTCTTTACCTCTGTGTG-3' (3' primer)(SEQ ID
NO:5)

where the small-case letters in the template oligo
indicate that at each position that a mixture of
reagents were used in synthesis by an amount of 62.5%
of the small case letter, and 12.5% each of the other
three nucleotides. Listed below the 6a sequence are
the sequences of 38 isolates cloned after six rounds of
SELEX performed with Rev protein with this population
of RNA. The differences found in these isolates from
the 6a sequences are indicated by bold-faced
characters. Underlined are the predicted base pairings
that comprise the bulge-flanking stems of the Motif I
Rev ligands. Bases that are included from the 5' and 3'

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fixed flanking sequences are lower case.

Figure 22 shows three sets of tabulations
containing:

5   A) The count of each nucleotide found at corresponsing
positions of the Rev 6a ligand sequence in the
collection of sequences found in Figure 21;

B) The fractional frequency of each nucleotide found
at these positions ($x + 38$, where x is the count from

10 1.); and

C) The difference between the fractional frequency of
B) and the expected frequency based on the input
mixture of oligonucleotides during template synthesis
[for "wild type" positions, ($x + 38$) - 0.625 and for

15 alternative sequences ($x + 38$) - 0.125].

Figure 23 shows three sets of tabulations
containing:

A) The count of each base pair found at corresponsing

20 positions of the Rev 6a ligand sequence in the
collection of sequences found in Figure 21,

B) The fractional frequency of each nucleotide found
at these positions ($x + 38$, where x is the count from
A),

25 C) The difference between the fractional frequency of
B) and the expected frequency based on the input
mixture of oligonucleotides during template synthesis
[for "wild type" positions, ($x + 38$) - 0.39; for base
pairs that contain one alternate nucleotide and one

30 wild type nucleotide, ($x + 38$) - 0.078; and for base
pairings of two alternate nucleotides ($x + 38$) -
0.016]. Values are shown for purine pyrimidine
pairings only, the other eight pyrimidine and purine
pairings are collectively counted and shown as "other"

35 and are computed for section C) as ($x + 38$) - 0.252.

Figure 24 shows the previously determined Rev

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protein ligand Motif I consensus (U.S. patent
application serial number 07/714,131 filed June 10,
1991; WO 91/19813). The 6a sequence from the same
application, and the preferred consensus derived from
5   the biased randomization SELEX as interpreted from the
data presented in Figures 22 and 23. Absolutely
conserved positions in the prefered consensus are shown
in bold face characters, and S-S' indicates either a C-
G or G-C base pair.
10

Figure 25 depicts the natural RNA sequence (or TAR
RNA) from HIV-1 with which the tat protein interacts.
The boxed region of the sequence identifies those
nucleotides that have been found to be important in the
15   tat-TAR interaction.

Figure 26 lists the sequences of ligands isolated
by the present invention as nucleic acid ligands to the
HIV-1 tat protein. The sequences are grouped according
20   to common secondary structures and primary sequence in
three motifs (I, II, and III). Inverted repeat
sequences that predict RNA helices are shown with
arrows. The regions of primary sequence homology
within each motif are outlined with dashed boxes. The
25   boundaries of the sequence information essential for
high affinity binding is indicated by a solid-lined
box. Sequences 1 and 17 do not fit into any of the
three identified motifs.

30   Figure 27 depicts a schematic diagram of the
consensus secondary structure and primary sequence of
each of the ligand motifs given in Figure 26. X
indicates non-conserved nucleotide positions. X'
indicates a base-pairing complement to X at that
35   position in a helix, R indicates purine and Y
pyrimidine. The dashed line in motif III indicates a
variable number of nucleotides at that portion of the

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loop.

    Figure 28 is the <u>tat</u> protein concentration-
dependent binding of selected ligand RNAs from Figure
 5   26, and the 40n RNA candidate mixture, to
nitrocellulose filters.

    FIGURE 29 depicts nucleotide sequences (SEQ ID
NO:137-155) of RNA ligands isolated by SELEX for the
10   human thrombin protein (Sigma). Each sequence is
divided into 3 blocks from left to right: 1) the 5'
fixed region, 2) the 30N variable region, and 3) the 3'
fixed region. Individual sequences are grouped into
class I and class II by conserved sequence motifs
15   within the 30N variable region as indicated by bold,
underlined characters.

    FIGURE 30 shows proposed secondary structures of
RNA ligands (SEQ ID NO: 156-159). (A) shows the
20   sequence of the 76 nucleotide class I RNA clones 6, 16,
and 18, and the class II 72 nucleotide clone 27. The
boundary determinations where [denotes a 5' boundary
and] denotes a 3' boundary are also shown. The
possible secondary structures of each RNA are shown in
25   (B) as determined from boundary experiments.
Boundaries are underlined. In (A) and (B) the 5' and
3' fixed regions are depicted by small case lettering,
the 30N random region by caps and the conserved region
by bold caps. The hairpin structures that were
30   synthesized are boxed with the total number of
nucleotides indicated.

    FIGURE 31 depicts binding curves for thrombin
ligands. In (A), RNAs with unique 30N sequence motifs
35   (see Fig. 29) were chosen for binding analysis with
human thrombin (Sigma), including the three from Class
I: RNA 6, RNA 16, and RNA 18, and one from Class II:

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RNA 27. Binding of bulk RNA sequences of the 30N3
candidate mixture is also shown. In (B), binding of
class I RNA clones 6, 16, 18 and class II RNA clone 27
is shown, but with human thrombin from Enzyme Research
5   Laboratories. In (C), binding of the 15mer ssDNA 5'-
GGTTGGTGTGGTTGG-3' (G15D) (SEQ ID NO:1), the class I
clone 16 hairpin structures (24R, 39D) and the class II
clone 27 hairpin structure (33R) (see Fig. 30B) are
shown under identical conditions as in (B). In the
10   case of the RNA hairpin structures, R denotes RNA
synthesis and D denotes transcription from a DNA
template.

       FIGURE 32 depicts a binding comparison of RNA
15   ligands between unmodified RNA and RNA with pyrimidines
modified to contain the 2'-NH_2 ribose nucleotide.
Binding comparisons of (A) bulk RNA 30N candidate
mixture and 2-NH_2 modified 30N candidate mixture, (B)
class I RNA 16 and 2-NH_2 modified RNA 16, and (C) class
20   II RNA 27 and 2-NH_2 modified RNA 27 are shown.

       FIGURE 33 depicts the competition experiments
between the 15mer ssDNA G15D and RNA hairpin ligands of
this invention for binding to human thrombin. In A)
25   concentration of the tracer G15D is equal to the
concentration of protein at 1 μM. The competitors for
binding include G15D itself, the 24 and 39 nucleotide
RNA hairpin structures from class I RNA 16, and the 33
nucleotide RNA hairpin structure from class II RNA 27
30   (see Figure 30). Binding is expressed as the relative
fraction G15D bound, which is the ratio of G15D binding
with competitor to G15D binding without competitor. In
B) the RNA 33 is the tracer and the concentration of
the tracer is equal to the concentration of protein at
35   300 nM. The competitors for binding include th ssDNA
G15D and RNA 24.

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FIGURE 34 shows the results of functional assays
of thrombin in the presence and absence of the RNA
ligand inhibitors described. In A) the hydrolysis by
thrombin of the chromogenic substrate S-2238 (H-D-Phe-
Pip-Arg-pNitroaniline) at the indicated thrombin and
RNA concentration was measured by the change in OD 405.
In B) the conversion of fibrinogen to fibrin and
resulting clot formation was measured by the tilt test
in the presence and absence of the RNA ligand
inhibitors described.

FIGURE 35 shows specificity of binding for
thrombin ligands. Class I RNA 16, class II RNA 27, and
bulk 30N3 RNA were chosen for binding analysis with A)
human antithrombin III (Sigma), and B) human
prothrombin (Sigma).

FIGURE 36 shows binding curves for family 1 ligand
7A (Δ), family 2 ligand 12A (□), random RNA, SELEX
experiment A(+) and random RNA, SELEX experiment B (x).
The fraction of RNA bound to nitrocellulose filters is
plotted as a function of free protein concentration and
data points were fitted to eq. 2. The following
concentrations of RNA were used: < 100 pM for 7A and
12A, and 10 nM for random RNAs. Binding reactions were
done at 37°C in phosphate buffered saline containing
0.01% human serum albumin.

FIGURE 37 shows the effect of RNA ligands 5A (o),
7A (Δ), 12A (□), 26A (◊), random RNA, SELEX experiment
A (+) and random RNA, SELEX experiment B (x) on binding
of ^{125}I-bFGF to the low-affinity (panel A) and the high-
affinity (panel B) cell-surface receptors. Experiments
were done essentially as described in Roghani &
Moscatelli (1992) J. Biol. Chem. <u>267</u>:22156.

FIGURE 38 shows the competitive displacement of

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32P-labeled RNA ligands 5A (o), 7A (Δ), 12A (□), and
26A (◊) by heparin (average molecular weight 5,000 Da).
Percent of total input RNA bound to nitrocellulose
filters is plotted as a function of heparin
5   concentration. Experiments were done at 37°C in
phosphate buffered saline containing 0.01% human serum
albumin, 0.3 uM RNA, and 30 nM bFGF.

FIGURE 39 shows the proposed secondary structures
10   for Family 1 ligands that bind to bFGF with high
affinity. Arrows indicate double stranded (stem)
regions that flank the conserved loop. Lower case
symbols indicate nucleotides in the constant region.

15   FIGURE 40 shows the proposed secondary structures
for Family 1 ligands.

FIGURE 41 shows the consensus structures for
Family 1 and Family 2 ligands. Y = C or U; R = A or G;
20   W = A or U; H = A, U, or C; D = A, G, or U; N = any
base. Complementary bases are primed. Symbols in
parenthesis indicate a variable number of bases or base
pairs at that position ranging within limits given in
the subscript.
25

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
This application is an extension and improvement
of the method for identifying nucleic acid ligands
referred to as SELEX. The SELEX method is described in
30   detail in U.S. patent application serial number
07/714,131 filed June 10, 1991 entitled Nucleic Acid
Ligands and 07/536,428 filed June 11, 1990 entitled
Systematic Evolution of Ligands by EXponential
Enrichment and in PCT Patent Application Publication WO
35   91/19813, published December 26, 1991 entitled Nucleic
Acid Ligands. The full text of these applications,
including but not limited to, all definitions and

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descriptions of the SELEX process, are specifically
incorporated herein by reference.

This application includes methods for identifying
and producing improved nucleic acid ligands based on
the basic SELEX process. The application includes
separate sections covering the following embodiments of
the invention: I. The SELEX Process; II. Techniques
for Identifying Improved Nucleic Acid Ligands
Subsequent to Performing SELEX; III. Sequential SELEX
Experiments - Walking; IV. Elucidation of Structure of
Ligands Via Covariance Analysis; V. Elucidation of an
Improved Nucleic Acid Ligand for HIV-RT (Example I);
VI. Performance of Walking Experiment With HIV-RT
Nucleic Acid Ligand to Identify Extended Nucleic Acid
Ligands; and VII. Elucidation of an Improved Nucleic
Acid Ligand for HIV-1 Rev Protein (Example II); Nucleic
Acid Ligands to the HIV-1 tat Protein (Example III);
Nucleic Acid Ligands to Thrombin (Example IV); and
Nucleic Acid Ligands to bFGF (Example V).

Improved nucleic acid ligands to the HIV-RT and
HIV-1 Rev proteins are disclosed and claimed herein.
This invention includes the specific nucleic acid
ligands identified herein. The scope of the ligands
covered by the invention extends to all ligands of the
HIV-RT and Rev proteins identified according to the
procedures described herein. More specifically, this
invention includes nucleic acid sequences that are
substantially homologous to and that have substantially
the same ability to bind the HIV-RT or Rev proteins,
under physiological conditions, as the nucleic acid
ligands identified herein. By substantially
homologous, it is meant, a degree of homology in excess
of 70%, most preferably in excess of 80%.
Substantially homologous also includes base pair flips
in those areas of the nucleic acid ligands that include
base pairing regions. Substantially the same ability
to bind the HIV-RT or Rev protein means that the

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affinity is within two orders of magnitude of the
affinity of the nucleic acid ligands described herein
and preferably within one order of magnitude. It is
well within the skill of those of ordinary skill in the
5.   art to determine whether a given sequence is
substantially homologous to and has substantially the
same ability to bind the HIV-RT or HIV-1 Rev protein as
the sequences identified herein.

10   I.   The SELEX Process.
            In its most basic form, the SELEX process may be
defined by the following series of steps:
            1)   A candidate mixture of nucleic acids of
differing sequence is prepared. The candidate mixture
15   generally includes regions of fixed sequences (i.e.,
each of the members of the candidate mixture contains
the same sequences in the same location) and regions of
randomized sequences. The fixed sequence regions are
selected either: a) to assist in the amplification
20   steps described below; b) to facilitate mimicry of a
sequence known to bind to the target; or c) to enhance
the concentration of a given structural arrangement of
the nucleic acids in the candidate mixture. The
randomized sequences can be totally randomized (i.e.,
25   the probability of finding a base at any position being
one in four) or only partially randomized (e.g., the
probability of finding a base at any location can be
selected at any level between 0 and 100 percent).
            2)   The candidate mixture is contacted with the
30   selected target under conditions favorable for binding
between the target and members of the candidate
mixture. Under these circumstances, the interaction
between the target and the nucleic acids of the
candidate mixture can be considered as forming nucleic
35   acid-target pairs between the target and the nucleic
acids having the strongest affinity for the target.
            3)   The nucleic acids with the highest affinity
for the target are partitioned from those nucleic acids

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with lesser affinity to the target. Because only an
extremely small number of sequences (and possibly only
one molecule of nucleic acid) corresponding to the
highest affinity nucleic acids exist in the candidate
mixture, it is generally desirable to set the
partitioning criteria so that a significant amount of
the nucleic acids in the candidate mixture
(approximately 5-50%) are retained during partitioning.

      4)   Those nucleic acids selected during
partitioning as having the relatively higher affinity
to the target are then amplified to create a new
candidate mixture that is enriched in nucleic acids
having a relatively higher affinity for the target.

      5)   By repeating the partitioning and amplifying
steps above, the newly formed candidate mixture
contains fewer and fewer unique sequences, and the
average degree of affinity of the nucleic acids to the
target will generally increase. Taken to its extreme,
the SELEX process will yield a candidate mixture
containing one or a small number of unique nucleic
acids representing those nucleic acids from the
original candidate mixture having the highest affinity
to the target molecule.

     The SELEX Patent Applications describe and
elaborate on this process in great detail. Included
are targets that can be used in the process; methods
for the preparation of the initial candidate mixture;
methods for partitioning nucleic acids within a
candidate mixture; and methods for amplifying
partitioned nucleic acids to generate enriched
candidate mixtures. The SELEX Patent Applications also
describe ligand solutions obtained to a number of
target species, including both protein targets wherein
the protein is and is not a nucleic acid binding
protein.

     SELEX delivers high affinity ligands of a target
molecule. This represents a singular achievement that

is unprecedented in the field of nucleic acids
research. The present invention is directed at methods
for taking the SELEX derived ligand solution in order
to develop novel nucleic acid ligands having the

5   desired characteristics. The desired characteristics
for a given nucleic acid ligand may vary. All nucleic
acid ligands are capable of forming a complex with the
target species. In some cases, it is desired that the
nucleic acid ligand will serve to inhibit one or more

10   of the biological activities of the target. In other
cases, it is desired that the nucleic acid ligand
serves to modify one or more of the biological
activities of the target. In other cases, the nucleic
acid ligand serves to identify the presence of the

15   target, and its effect on the biological activity of
the target is irrelevant.

II.   Techniques for Identifying Improved Nucleic Acid
      Ligands Subsequent to Performing SELEX.

20   In order to produce nucleic acids desirable for
use as a pharmaceutical, it is preferred that the
nucleic acid ligand 1) binds to the target in a manner
capable of achieving the desired effect on the target;
2) be as small as possible to obtain the desired

25   effect; 3) be as stable as possible; and 4) be a
specific ligand to the chosen target. In most, if not
all, situations it is preferred that the nucleic acid
ligand have the highest possible affinity to the
target. Modifications or derivatizations of the ligand

30   that confer resistance to degradation and clearance in
situ during therapy, the capability to cross various
tissue or cell membrane barriers, or any other
accessory properties that do not significantly
interfere with affinity for the target molecule may

35   also be provided as improvements. The present
invention includes the methods for obtaining improved
nucleic acid ligands after SELEX has been performed.

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Assays of ligand effects on target molecule
function. One of the uses of nucleic acid ligands
derived by SELEX is to find ligands that alter target
molecule function. Because ligand analysis requires
5   much more work than is encountered during SELEX
enrichments, it is a good procedure to first assay for
inhibition or enhancement of function of the target
protein. One could even perform such functional tests
of the combined ligand pool prior to cloning and
10   sequencing. Assays for the biological function of the
chosen target are generally available and known to
those skilled in the art, and can be easily performed
in the presence of the nucleic acid ligand to determine
if inhibition occurs.
15   Affinity assays of the ligands. SELEX enrichment
will supply a number of cloned ligands of probable
variable affinity for the target molecule. Sequence
comparisons may yield consensus secondary structures
and primary sequences that allow grouping of the ligand
20   sequences into motifs. Although a single ligand
sequence (with some mutations) can be found frequently
in the total population of cloned sequences, the degree
of representation of a single ligand sequence in the
cloned population of ligand sequences may not
25   absolutely correlate with affinity for the target
molecule. Therefore mere abundance is not the sole
criterion for judging "winners" after SELEX and binding
assays for various ligand sequences (adequately
defining each motif that is discovered by sequence
30   analysis) are required to weigh the significance of the
consensus arrived at by sequence comparisons. The
combination of sequence comparison and affinity assays
should guide the selection of candidates for more
extensive ligand characterization.
35   Information boundaries determination. An
important avenue for narrowing down what amount of
sequence is relevant to specific affinity is to

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establish the boundaries of that information within a
ligand sequence. This is conveniently accomplished by
selecting end-labeled fragments from hydrolyzed pools
of the ligand of interest so that 5' and 3' boundaries
5   of the information can be discovered. To determine a
3' boundary, one performs a large-scale _in vitro_
transcription of the PCR ligand, gel purifies the RNA
using UV shadowing on an intensifying screen,
phosphatases the purified RNA, phenol extracts
10   extensively, labels by kinasing with ^{32}P, and gel
purifies the labeled product (using a film of the gel
as a guide). The resultant product may then be
subjected to pilot partial digestions with RNase T1
(varying enzyme concentration and time, at 50°C in a
15   buffer of 7 M urea, 50 mM NaCitrate pH 5.2) and
alkaline hydrolysis (at 50 mM $NaCO_3$, adjusted to pH
9.0 by prior mixing of 1M bicarbonate and carbonate
solutions; test over ranges of 20 to 60 minutes at
95°C). Once optimal conditions for alkaline hydrolysis
20   are established (so that there is an even distribution
of small to larger fragments) one can scale up to
provide enough material for selection by the target
(usually on nitrocellulose filters). One then sets up
binding assays, varying target protein concentration
25   from the lowest saturating protein concentration to
that protein concentration at which approximately 10%
of RNA is bound as determined by the binding assays for
the ligand. One should vary target concentration (if
target supplies allow) by increasing volume rather than
30   decreasing absolute amount of target; this provides a
good signal to noise ratio as the amount of RNA bound
to the filter is limited by the absolute amount of
target. The RNA is eluted as in SELEX and then run on
a denaturing gel with T1 partial digests so that the
35   positions of hydrolysis bands can be related to the
ligand sequence.
       The 5' boundary can be similarly determined.

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Large-scale _in vitro_ transcriptions are purified as
described above. There are two methods for labeling
the 3' end of the RNA.   One method is to kinase Cp
with ^{32}P (or purchase ^{32}P-Cp) and ligate to the purified
RNA with RNA ligase. The labeled RNA is then purified
as above and subjected to very identical protocols. An
alternative is to subject unlabeled RNAs to partial
alkaline hydrolyses and extend an annealed, labeled
primer with reverse transcriptase as the assay for band
positions. One of the advantages over pCp labeling is
the ease of the procedure, the more complete sequencing
ladder (by dideoxy chain termination sequencing) with
which one can correlate the boundary, and increased
yield of assayable product. A disadvantage is that the
extension on eluted RNA sometimes contains artifactual
stops, so it may be important to control by spotting
and eluting starting material on nitrocellulose filters
without washes and assaying as the input RNA.

The result is that it is possible to find the
boundaries of the sequence information required for
high affinity binding to the target.

An instructive example is the determination of the
boundaries of the information found in the nucleic acid
ligand for HIV-RT. (See, U.S. patent application
serial number 07/714,131 filed June 10, 1991 and PCT
Patent Application Publication WO 91/19813, published
December 26, 1991 entitled Nucleic Acid Ligands).
These experiments are described in detail below. The
original pool of enriched RNAs yielded a few specific
ligands for HIV-RT (one ligand, 1.1, represented 1/4 of
the total population, nitrocellulose affinity sequences
represented 1/2 and some RNAs had no affinity for
either). Two high-affinity RT ligands shared the
sequence ...UUCCGNNNNNNNNNCGGGAAAA.... (SEQ ID NO:6)
Boundary experiments of both ligands established a
clear 3' boundary and a less clear 5' boundary. It can
be surmised from the boundary experiments and secondary

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SELEX experiments that the highest affinity ligands
contained the essential information
UCCGNNNNNNNNNCGGGAAAAN'N'N'N' (SEQ ID NO:7)(where N's
base pair to Ns in the 8 base loop sequence of the
hairpin formed by the pairing of UCCG to CGGG) and that
the 5' U would be dispensable with some small loss in
affinity. In this application, the construction of
model compounds confirmed that there was no difference
in the affinity of sequences with only one 5' U
compared to 2 5' U's (as is shared by the two compared
ligands), that removal of both U's caused a 5-fold
decrease in affinity and of the next C a more drastic
loss in affinity. The 3' boundary which appeared to be
clear in the boundary experiments was less precipitous.
This new information can be used to deduce that what is
critical at the 3' end is to have at least three
base-paired nucleotides (to sequences that loop between
the two strands of Stem 1). Only two base-paired
nucleotides result in a 12-fold reduction in affinity.
Having no 3' base-paired nucleotides (truncation at the
end of Loop 2) results in an approximately 70-fold
reduction in affinity.

       Quantitative and qualitative assessment of
individual nucleotide contributions to affinity.

       SECONDARY SELEX. Once the minimal high affinity
ligand sequence is identified, it may be useful to
identify the nucleotides within the boundaries that are
crucial to the interaction with the target molecule.
One method is to create a new random template in which
all of the nucleotides of a high affinity ligand
sequence are partially randomized or blocks of
randomness are interspersed with blocks of complete
randomness. Such "secondary" SELEXes produce a pool of
ligand sequences in which crucial nucleotides or
structures are absolutely conserved, less crucial
features preferred, and unimportant positions unbiased.
Secondary SELEXes can thus help to further elaborate a

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consensus that is based on relatively few ligand
sequences. In addition, even higher-affinity ligands
may be provided whose sequences were unexplored in the
original SELEX.

5   In this application we show such a biased
randomization for ligands of the HIV-1 Rev protein. In
U.S. patent application serial number 07/714,131 filed
June 10, 1991, and PCT Patent Application Publication
WO 91/19813, published December 26, 1991 entitled
10   Nucleic Acid Ligands, nucleic acid ligands to the HIV-1
Rev protein were described. One of these ligand
sequences bound with higher affinity than all of the
other ligand sequences (Rev ligand sequence 6a, shown
in Figure 12) but existed as only two copies in the 53
15   isolates that were cloned and sequenced. In this
application, this sequence was incorporated in a
secondary SELEX experiment in which each of the
nucleotides of the 6a sequence (confined to that part
of the sequence which comprises a Rev protein binding
20   site defined by homology to others of Rev ligand motif
I) was mixed during oligonucleotide synthesis with the
other three nucleotides in the the ratio
62.5:12.5:12.5:12.5. For example, when the sequence
at position G1 is incorporated during oligo synthesis,
25   the reagents for G,A,T, and C are mixed in the ratios
62.5:12.5:12.5:12.5. After six rounds of SELEX using
the Rev protein, ligands were cloned from this mixture
so that a more comprehensive consensus description
could be derived.
30   NUCLEOTIDE SUBSTITUTION. Another method is to
test oligo-transcribed variants where the SELEX
consensus may be confusing. As shown above, this has
helped us to understand the nature of the 5' and 3'
boundaries of the information required to bind HIV-RT.
35   As is shown in the attached example this has helped to
quantitate the consensus of nucleotides within Stem 1
of the HIV-RT pseudoknot.

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CHEMICAL MODIFICATION. Another useful set of
techniques are inclusively described as chemical
modification experiments. Such experiments may be used
to probe the native structure of RNAs, by comparing
5   modification patterns of denatured and non-denatured
states. The chemical modification pattern of an RNA
ligand that is subsequently bound by target molecule
may be different from the native pattern, indicating
potential changes in structure upon binding or
10   protection of groups by the target molecule. In
addition, RNA ligands will fail to be bound by the
target molecule when modified at positions crucial to
either the bound structure of the ligand or crucial to
interaction with the target molecule. Such experiments
15   in which these positions are identified are described
as "chemical modification interference" experiments.
There are a variety of available reagents to
conduct such experiments that are known to those
skilled in the art (see, Ehresmann et al. (1987) Nuc.
20   Acids. Res. 15:9109). Chemicals that modify bases can
be used to modify ligand RNAs. A pool is bound to the
target at varying concentrations and the bound RNAs
recovered (much as in the boundary experiments) and the
eluted RNAs analyzed for the modification. Assay can
25   be by subsequent modification-dependent base removal
and aniline scission at the baseless position or by
reverse transcription assay of sensitive (modified)
positions. In such assays bands (indicating modified
bases) in unselected RNAs appear that disappear
30   relative to other bands in target protein-selected
RNAs. Similar chemical modifications with
ethylnitrosourea, or via mixed chemical or enzymatic
synthesis with, for example, 2'-methoxys on ribose or
phosphorothioates can be used to identify essential
35   atomic groups on the backbone. In experiments with
2'-methoxy vs. 2'-OH mixtures, the presence of an
essential OH group results in enhanced hydrolysis

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relative to other positions in molecules that have been
stringently selected by the target.

An example of how chemical modification can be
used to yield useful information about a ligand and
help efforts to improve its functional stability is
given below for HIV-RT. Ethylnitrosourea modification
interference identified 5 positions at which
modification interfered with binding and 2 of those
positions at which it interfered drastically.
Modification of various atomic groups on the bases of
the ligand were also identified as crucial to the
interaction with HIV-RT. Those positions were
primarily in the 5' helix and bridging loop sequence
that was highly conserved in the SELEX phylogeny (Stem
I and Loop 2, Figure 1). These experiments not only
confirmed the validity of that phylogeny, but informed
ongoing attempts to make more stable RNAs. An RT
ligand was synthesized in which all positions had
2'-methoxy at the ribose portions of the backbone.
This molecule bound with drastically reduced affinity
for HIV-RT. Based on the early modification
interference experiments and the SELEX phylogeny
comparisons, it could be determined that the 3' helix
(Stem II Fig. 1) was essentially a structural component
of the molecule. A ligand in which the 12 ribose
residues of that helix were 2'-methoxy was then
synthesized and it bound with high affinity to HIV-RT.
In order to determine if any specific 2'-OHs of the
remaining 14 residues were specifically required for
binding, a molecule in which all of the riboses of the
pseudoknot were synthesized with mixed equimolar
(empirically determined to be optimal) reagents for
2'-OH and 2'-methoxy formation. Selection by HIV-RT
from this mixture followed by alkaline hydrolysis
reveals bands of enhanced hydrolysis indicative of
predominating 2' hydroxyls at those positions.
Analysis of this experiment lead to the conclusion that

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residues (G4, A5, C13 and G14) must have 2'-OH for high
affinity binding to HIV-RT.

Comparisons of the intensity of bands for bound
and unbound ligands may reveal not only modifications
that interfere with binding, but also modifications
that enhance binding. A ligand may be made with
precisely that modification and tested for the enhanced
affinity. Thus chemical modification experiments can
be a method for exploring additional local contacts
with the target molecule, just as "walking" (see below)
is for additional nucleotide level contacts with
adjacent domains.

One of the products of the SELEX procedure is a
consensus of primary and secondary structures that
enables the chemical or enzymatic synthesis of
oligonucleotide ligands whose design is based on that
consensus. Because the replication machinery of SELEX
requires that rather limited variation at the subunit
level (ribonucleotides, for example), such ligands
imperfectly fill the available atomic space of a target
molecule's binding surface. However, these ligands can
be thought of as high-affinity scaffolds that can be
derivatized to make additional contacts with the target
molecule. In addition, the consensus contains atomic
group descriptors that are pertinent to binding and
atomic group descriptors that are coincidental to the
pertinent atomic group interactions. For example, each
ribonucleotide of the pseudoknot ligand of HIV-RT
contains a 2' hydroxyl group on the ribose, but only
two of the riboses of the pseudoknot ligand cannot be
substituted at this position with 2'-methoxy. A
similar experiment with deoxyribonucleotide mixtures
with ribonucleotide mixtures (as we have done with 2'-
methoxy and 2' hydroxy mixtures) would reveal which
riboses or how many riboses are dispensable for binding
HIV-RT. A similar experiment with more radical
substitutions at the 2' position would again reveal the

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allowable substitutions at 2' positions. One may
expect by this method to find derivatives of the
pseudoknot ligand that confer higher affinity
association with HIV-RT. Such derivatization does not
5   exclude incorporation of cross-linking agents that will
give specifically directly covalent linkages to the
target protein. Such derivatization analyses are not
limited to the 2' position of the ribose, but could
include derivatization at any position in the base or
10   backbone of the nucleotide ligand.
        A logical extension of this analysis is a
situation in which one or a few nucleotides of the
polymeric ligand is used as a site for chemical
derivative exploration. The rest of the ligand serves
15   to anchor in place this monomer (or monomers) on which
a variety of derivatives are tested for non-
interference with binding and for enhanced affinity.
Such explorations may result in small molecules that
mimic the structure of the initial ligand framework,
20   and have significant and specific affinity for the
target molecule independent of that nucleic acid
framework. Such derivatized subunits, which may have
advantages with respect to mass production, therapeutic
routes of administration, delivery, clearance or
25   degradation than the initial SELEX ligand, may become
the therapeutic and may retain very little of the
original ligand. This approach is thus an additional
utility of SELEX. SELEX ligands can allow directed
chemical exploration of a defined site on the target
30   molecule known to be important for the target function.
        Structure determination. These efforts have
helped to confirm and evaluate the sequence and
structure dependent association of ligands to HIV-RT.
Additional techniques may be performed to provide
35   atomic level resolution of ligand/target molecule
complexes. These are NMR spectroscopy and X-ray
crystallography. With such structures in hand, one can

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then perform rational design as improvements on the
evolved ligands supplied by SELEX. The computer
modeling of nucleic acid structures is described below.

Chemical Modification. This invention includes
5   nucleic acid ligands wherein certain chemical
modifications have been made in order to increase the
in vivo stability of the ligand or to enhance or
mediate the delivery of the ligand. Examples of such
modifications include chemical substitutions at the
10   ribose and/or phosphate positions of a given RNA
sequence. See, e.g., Cook, et al. PCT Application WO
9203568; U.S. Patent No. 5,118,672 of Schinazi et al.;
Hobbs et al. (1973) Biochem. 12:5138; Guschlbauer et
al. (1977) Nucleic Acids Res. 4:1933; Shibaharu et al.
15   Nucl. Acids. Res. (1987) 15:4403; Pieken et al. (1991)
Science 253:314, each of which is specifically
incorporated herein by reference.

III.   Sequential SELEX Experiments - Walking.
20   In one embodiment of this invention, after a
minimal consensus ligand sequence has been determined
for a given target, it is possible to add random
sequence to the minimal consensus ligand sequence and
evolve additional contacts with the target, perhaps to
25   separate but adjacent domains. This procedure is
referred to as "walking" in the SELEX Patent
Applications. The successful application of the
walking protocol is presented below to develop an
enhanced binding ligand to HIV-RT.
30   The walking experiment involves two SELEX
experiments performed sequentially. A new candidate
mixture is produced in which each of the members of the
candidate mixture has a fixed nucleic acid region that
corresponds to a SELEX-derived nucleic acid ligand.
35   Each member of the candidate mixture also contains a
randomized region of sequences. According to this
method it is possible to identify what are referred to

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as "extended" nucleic acid ligands, that contain
regions that may bind to more than one binding domain
of a target.

5   IV.   Elucidation of Structure of Ligands Via Covariance
              Analysis.
              In conjunction with the empirical methods for
        determining the three dimensional structure of nucleic
        acids, the present invention includes computer modeling
10   methods for determining structure of nucleic acid
        ligands.
              Secondary structure prediction is a useful guide
        to correct sequence alignment. It is also a highly
        useful stepping-stone to correct 3D structure
15   prediction, by constraining a number of bases into A-
        form helical geometry.
              Tables of energy parameters for calculating the
        stability of secondary structures exist. Although
        early secondary structure prediction programs attempted
20   to simply maximize the number of base-pairs formed by a
        sequence, most current programs seek to find structures
        with minimal free energy as calculated by these
        thermodynamic parameters. There are two problems in
        this approach. First, the thermodynamic rules are
25   inherently inaccurate, typically to 10% or so, and
        there are many different possible structures lying
        within 10% of the global energy minimum. Second, the
        actual secondary structure need not lie at a global
        energy minimum, depending on the kinetics of folding
30   and synthesis of the sequence. Nonetheless, for short
        sequences, these caveats are of minor importance
        because there are so few possible structures that can
        form.
              The brute force predictive method is a dot-plot:
35   make an N by N plot of the sequence against itself, and
        mark an X everywhere a basepair is possible. Diagonal
        runs of X's mark the location of possible helices.

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Exhaustive tree-searching methods can then search for
all possible arrangements of compatible (i.e., non-
overlapping) helices of length L or more; energy
calculations may be done for these structures to rank
them as more or less likely. The advantages of this
method are that all possible topologies, including
pseudoknotted conformations, may be examined, and that
a number of suboptimal structures are automatically
generated as well. The disadvantages of the method are
that it can run in the worst cases in time proportional
to an exponential factor of the sequence size, and may
not (depending on the size of the sequence and the
actual tree search method employed) look deep enough to
find a global minimum.

    The elegant predictive method, and currently the
most used, is the Zuker program (Zuker (1989) Science
244:48). Originally based on an algorithm developed by
Ruth Nussinov, the Zuker program makes a major
simplifying assumption that no pseudoknotted
conformations will be allowed. This permits the use of
a dynamic programming approach which runs in time
proportional to only N^3 to N^4, where N is the length of
the sequence. The Zuker program is the only program
capable of rigorously dealing with sequences of than a
few hundred nucleotides, so it has come to be the most
commonly used by biologists. However, the inability of
the Zuker program to predict pseudoknotted
conformations is a fatal flaw, in that several
different SELEX experiments so far have yielded
pseudoknotted RNA structures, which were recognized by
eye. A brute-force method capable of predicting
pseudoknotted conformations must be used.

    The central element of the comparative sequence
analysis of the present invention is sequence
covariations. A covariation is when the identity of
one position depends on the identity of another
position; for instance, a required Watson-Crick base

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pair shows strong covariation in that knowledge of one
of the two positions gives absolute knowledge of the
identity at the other position. Covariation analysis
has been used previously to predict the secondary

5   structure of RNAs for which a number of related
sequences sharing a common structure exist, such as
tRNA, rRNAs, and group I introns. It is now apparent
that covariation analysis can be used to detect
tertiary contacts as well.

10   Stormo and Gutell have designed and implemented an
algorithm that precisely measures the amount of
covariations between two positions in an aligned
sequence set. The program is called "MIXY" - Mutual
Information at position X and Y.

15   Consider an aligned sequence set. In each column
or position, the frequency of occurrence of A, C, G, U,
and gaps is calculated. Call this frequency $f(b_x)$, the
frequency of base b in column x. Now consider two
columns at once. The frequency that a given base b

20   appears in column x is $f(b_x)$ and the frequency that a
given base b appears in column y is $f(b_y)$. If position
x and position y do not care about each other's
identity - that is, the positions are independent;
there is no covariation - the frequency of observing

25   bases b_x and b_y at position x and y in any given
sequence should be just $f(b_x b_y) = f(b_x)f(b_y)$. If there
are substantial deviations of the observed frequencies
of pairs from their expected frequencies, the positions
are said to covary. The amount of deviation from

30   expectation may be quantified with an information
measure M(x,y), the mutual information of x and y:

$$M(x,y) = \sum_{b_x b_y} f(b_x b_y) \ln \frac{f(b_x b_y)}{f(b_x)f(b_y)}$$

35

M(x,y) can be described as the number of bits of
information one learns about the identity of position y
from knowing just the identity of position y from

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knowing just the identity of position x. If there is
no covariation, M(x,y) is zero; larger values of M(x,y)
indicate strong covariation.

These numbers correlated extremely well to a
probability for close physical contact in the tertiary
structure, when this procedure was applied to the tRNA
sequence data set. The secondary structure is
extremely obvious as peaks in the M(x,y) values, and
most of the tertiary contacts known from the crystal
structure appear as peaks as well.

These covariation values may be used to develop
three-dimensional structural predictions.

In some ways, the problem is similar to that of
structure determination by NMR. Unlike
crystallography, which in the end yields an actual
electron density map, NMR yields a set of interatomic
distances. Depending on the number of interatomic
distances one can get, there may be one, few, or many
3D structures with which they are consistent.
Mathematical techniques had to be developed to
transform a matrix of interatomic distances into a
structure in 3D space. The two main techniques in use
are distance geometry and restrained molecular
dynamics.

Distance geometry is the more formal and purely
mathematical technique. The interatomic distances are
considered to be coordinates in an N-dimensional space,
where N is the number of atoms. In other words, the
"position" of an atom is specified by N distances to
all the other atoms, instead of the three (x,y,z) that
we are used to thinking about. Interatomic distances
between every atom are recorded in an N by N distance
matrix. A complete and precise distance matrix is
easily transformed into a 3 by N Cartesian coordinates,
using matrix algebra operations. The trick of distance
geometry as applied to NMR is dealing with incomplete
(only some of the interatomic distances are known) and

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imprecise data (distances are known to a precision of
only a few angstroms at best). Much of the time of
distance geometry-based structure calculation is thus
spent in pre-processing the distance matrix,

5   calculating bounds for the unknown distance values
based on the known ones, and narrowing the bounds on
the known ones. Usually, multiple structures are
extracted from the distance matrix which are consistent
with a set of NMR data; if they all overlap nicely, the

10   data were sufficient to determine a unique structure.
Unlike NMR structure determination, covariance gives
only imprecise distance values, but also only
probabilistic rather than absolute knowledge about
whether a given distance constraint should be applied.

15   Restrained molecular dynamics is a more ad hoc
procedure. Given an empirical force field that
attempts to describe the forces that all the atoms feel
(van der Waals, covalent bonding lengths and angles,
electrostatics), one can simulate a number of

20   femtosecond time steps of a molecule's motion, by
assigning every atom at a random velocity (from the
Boltzmann distribution at a given temperature) and
calculating each atom's motion for a femtosecond using
Newtonian dynamical equations; that is "molecular

25   dynamics". In restrained molecular dynamics, one
assigns extra ad hoc forces to the atoms when they
violate specified distance bounds.

            In the present case, it is fairly easy to deal
with the probabilistic nature of data with restrained

30   molecular dynamics. The covariation values may be
transformed into artificial restraining forces between
certain atoms for certain distance bounds; varying the
magnitude of the force according to the magnitude of
the covariance.

35   NMR and covariance analysis generates distance
restraints between atoms or positions, which are
readily transformed into structures through distance

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geometry or restrained molecular dynamics. Another
source of experimental data which may be utilized to
determine the three dimensional structures of nucleic
acids is chemical and enzymatic protection experiments,
which generate solvent accessibility restraints for
individual atoms or positions.

V.   ELUCIDATION OF AN IMPROVED NUCLEIC ACID LIGAND FOR
      HIV-RT.
         An example of the methods of the present invention
are presented herein for the nucleic acid ligand for
HIV-1 reverse transcriptase (HIV-RT). U.S. Patent
Application Serial No. 07/714,131 and PCT Patent
Application Publication WO 91/19813, published December
26, 1991 entitled Nucleic Acid Ligands describes the
results obtained when SELEX was performed with the HIV-
RT target. Inspection of the nucleic acid sequences
that were found to have a high affinity to HIV-RT, it
was concluded that the nucleic acid ligand solution was
configured as a pseudoknot.
         Described herein are experiments which establish
the minimum number of sequences necessary to represent
the nucleic acid ligand solution via boundary studies.
Also described are the construction of variants of the
ligand solution which are used to evaluate the
contributions of individual nucleotides in the solution
to the binding of the ligand solution to HIV-RT. Also
described is the chemical modification of the ligand
solution; 1) to corroborate its predicted pseudoknot
structure; 2) to determine which modifiable groups are
protected from chemical attack when bound to HIV-RT (or
become unprotected during binding); and 3) to determine
what modifications interfere with binding to HIV-RT
(presumably by modification of the three dimensional
structure of the ligand solution) and, therefore, which
are presumably involved in the proximal contacts with
the target.
         The nucleic acid ligand solution previously

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determined is shown in Figure 1. Depicted is an RNA
pseudoknot in which Stem 1 (as labeled) is conserved
and Stem 2 is relatively non-conserved; X indicates no
conservation and X' base-pairs to X. In the original
5   SELEX consensus U1 was preferred (existing at this
relative position in 11 of the 18 sequences that
contributed to the consensus), but A1 was also found
frequently (in 6 of the 18). There were two sequences
in which C-G was substituted for the base-pair of
10   G4-C13 and one A-U substitution. The preferred number
of nucleotides connecting the two strands of Stem 1 was
eight (in 8 of 18). The number and pattern of
base-paired nucleotides comprising Stem 2 and the
preference for A5 and A12 were derived from the
15   consensus of a secondary SELEX in which the random
region was constructed as follows
NNUUCCGNNNNNNNNNCGGGAAAANNNN (SEQ ID NO:8)(Ns are
randomized). One of the ligands was found to
significantly inhibit HIV-RT and failed to inhibit AMV
20   or MMLV reverse transcriptases.

     <u>Refinement of the information boundaries</u>. The
first two SELEX experiments in which 32 nucleotide
positions were randomized provided high affinity
ligands in which there was variable length for Stem 1
25   at its 5' end; that is, some ligands had the sequence
UUCCG which could base pair to CGGGA, UCCG to CGGG or
CCG to CGG. Determination of the boundaries of the
sequences donating high-affinity to the interaction
with HIV-RT was accomplished by selection from partial
30   alkaline hydrolysates of end-labeled clonal RNAs, a
rapid but qualitative analysis which suggested that the
highest affinity ligands contained the essential
information UCCGNNNNNNNNNCGGGAAAANN'N'N'(SEQ ID
NO:7)(where N's base pair to Ns in the 8 base loop
35   sequence of the hairpin formed by the pairing of UCCG
to CGGG) and that the 5' U would be dispensable with
some small loss in affinity. In order to more

stringently test the 5' sequences in a homogeneous
context, the binding experiments depicted in Figure 2
were performed. The RNA's transcribed from
oligonucleotide templates were all the same as the
complete sequence shown in the upper right hand corner
of the figure, except for the varying 5' ends as shown
in the boxes A-E lining the left margin. The result is
that one 5' U is sufficient for the highest-affinity
binding to HIV-RT (boxes A and B), that with no U there
is reduced binding (box C), and that any further
removal of 5' sequences reduces binding to that of
non-specific sequences (box D). The design (hereafter
referred to as ligand B) with only one 5' U (U1) was
used for the rest of the experiments described here.

Dependence on the length of Stem 2 was also
examined by making various 3' truncations at the 3' end
of ligand B. Deletion of as many as 3 nucleotides from
the 3' end (A24-U26) made no difference in affinity of
the molecule for HIV-RT. Deletion of the 3'-terminal 4
nucleotides (C23-U26) resulted in 7-fold reduced
binding, of 5 (G22-U26) resulted in approximately
12-fold reduction and of 6 nucleotides (U21-U26, or no
3' helix) an approximately 70-fold reduction in
affinity. Such reductions were less drastic than
reductions found for single-base substitutions reported
below, suggesting (with other data reported below) that
this helix serves primarily a structural role that aids
the positioning of crucial groups in Loop 2.

Testing the SELEX consensus for Stem 1. Various
nucleotide substitutions in the conserved Stem 1 were
prepared and their affinity to HIV-RT determined. As
shown in Figure 3, substitution of an A for U1 in model
RNAs made little difference in affinity for HIV-RT. C
(which would increase the stability of Stem1) or G
(represented by the U deletion experiment above) at
this position resulted in approximately 20-fold
lowering in affinity. Substitution of A for G16 (which

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would base-pair to U1) abolished specific binding. A
G-C pair was substituted for C2-G15 which also
abolished binding and for C3-G14 which reduced binding
about 10-fold. These two positions were highly

5   conserved in the phylogeny of SELEX ligands. Various
combinations were substituted for the G4-C13 base pair.
The order of affect of these on affinity were
G4-C13=C-G>U-A>A-U>>>>A-C where A-U is about 20-fold
reduced in affinity compared to G4-C13 and A-C is at

10   least 100- fold reduced. These results are consistent
with the SELEX consensus determined previously.

      Chemical probing of the pseudoknot structure. A
number of chemical modification experiments were
conducted to probe the native structure of ligand B, to

15   identify chemical modifications that significantly
reduced affinity of ligand B for HIV-RT, and to
discover changes in structure that may accompany
binding by HIV-RT. The chemicals used were
ethylnitrosourea (ENU) which modifies phosphates,

20   dimethyl sulfate (DMS) which modifies the base-pairing
faces of C (at N3) and A (at N1), carbodiimide (CMCT)
which modifies the base-pairing face of U (at N3) and
to some extent G (at N1), diethylpyrocarbonate (DEPC)
which modifies N7 of A and to a lesser extent the N7 of

25   G, and kethoxal which modifies the base-pairing N1 and
N2 of G. Most of the assays of chemical modification
were done on a ligand B sequence which was lengthened
to include sequences to which a labeled primer could be
annealed and extended with AMV reverse transcriptase.

30   Assay of ENU or DEPC modified positions were done on
ligand B by respective modification-dependent
hydrolysis, or modified base removal followed by
aniline scission of the backbone at these sites.

      The results of probing the native structure as

35   compared to modification of denatured ligand B are
summarized in Figure 4. The pattern of ENU
modification was not different between denatured native

states of the ligand suggesting that there is no stable
involvement of the phosphates or N7 positions of
purines in the solution structure of the pseudoknot.
The other modification data suggest that Stem 2 forms
5   rather stably and is resistant to any chemical
modifications affecting the base-pairs shown, although
the terminal A6-U26 is somewhat sensitive to
modification indicating equilibration between
base-paired and denatured states at this position. The
10   single-stranded As (A5, A17, A18, A19, and A20) are
fully reactive with DMS although A5, A19, and A20 are
diminished in reactivity to DEPC. The base-pairs of
Stem 1 seem to exhibit a gradation of resistance to
modification such that G4-C13>C3-G14>C2-G15>U1-G16
15   where G4-C13 is completely resistant to chemical
modification and U1-G16 is highly reactive. This
suggests that this small helix of the pseudoknot
undergoes transient and directional denaturation or
"fraying".

20   Protection of ligand B from chemical modification
by HIV-RT. Binding of protein changes the fraying
character of Helix I as shown in Figure 5 either by
stabilizing or protecting it. The natively reactive U1
is also protected upon binding. Binding of protein
25   increases the sensitivity of the base-pair A6-U26
suggesting that this is unpaired in the bound state.
This may be an indication of insufficient length of a
single nucleotide Loop I during binding, either because
it cannot bridge the bound Stem 1 to the end of Stem 2
30   in the native pseudoknot recognized by RT or because
binding increases the length requirement of Loop I by
changing the conformation from the native state. A17
and A19 of Loop II are also protected by binding to
HIV-RT. In addition, the single base bridge A12 is
35   protected upon binding.

        Modification interference studies of the RT ligand
B. The RNA ligand B was partially modified (with all of

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the chemicals mentioned above for structure
determination). This modified population was bound
with varying concentrations of the protein, and the
bound species were assayed for the modified positions.
From this, it can be determined where modification
interferes with binding, and where there is no or
little effect. A schematic diagram summarizing these
modification interference results is shown in Figure 6.
As shown, most of the significant interference with
binding is clustered on the left hand side of the
pseudoknot which contains the Stem 1 and Loop 2. This
is also the part of the molecule that was highly
conserved (primary sequence) in the collection of
sequences isolated by SELEX and where substitution
experiments produced the most drastic reduction in
binding affinity to HIV-RT.

Substitution of 2'-methoxy for 2'-hydroxyl on
riboses of ligand B. "RNA" molecules in which there is
a 2'-methoxy bonded to the 2' carbon of the ribose
instead of the normal hydroxyl group are resistant to
enzymatic and chemical degradation. In order to test
how extensively 2'-methoxys can be substituted for
2'-OH's in RT ligands, four oligos were prepared as
shown in Figure 7. Because fully substituted
2'-methoxy ligand binds poorly (ligand D), and because
we had found that most of the modification interference
sites were clustered at one end of the pseudoknot,
subsequent attempts to substitute were confined to the
non-specific 3' helix as shown in boxes B and C. Both
of these ligands bind with high affinity to HIV-RT.
Oligonucleotides were then prepared in which the
allowed substitutions at the ribose of Stem 2 were all
2'-methoxy as in C of Figure 7 and at the remaining 14
positions mixed synthesis were done with 2'-methoxy and
2'-OH phosphoramidite reagents. These oligos were
subjected to selection by HIV-RT followed by alkaline
hydrolysis of selected RNAs and gel separation

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(2'-methoxys do not participate in alkaline hydrolysis
as do 2'-hydroxyls). As judged by visual inspection of
films (see Figure 8) and quantitative determination of
relative intensities using an Ambis detection system
5   (see Example below for method of comparison), the
ligands selected by HIV-RT from the mixed incorporation
populations showed significantly increased hydrolysis
at positions C13 and G14 indicating interference by
2'-methoxys at these positions. In a related
10   experiment where mixtures at all positions were
analyzed in this way, G4, A5, C13 and G14 showed 2' 0-
methyl interference.
       The results of substitution experiments,
quantitative boundary experiments and chemical probing
15   experiments are highly informative about the nature of
the pseudoknot inhibitor of HIV-RT and highlight
crucial regions of contact on this RNA. These results
are provided on a nucleotide by nucleotide basis below.
       U1 can be replaced with A with little loss in
20   affinity but not by C or G. Although U1 probably makes
transient base-pairing to G16, modification of U1-N3
with CMCT does not interfere with binding to HIV-RT.
However, binding by HIV-RT protects the N3 of U1
perhaps by steric or electrostatic shielding of this
25   position. Substitution with C which forms a more
stable base-pair with G16 reduces affinity.
Replacement of G16 with A which forms a stable U1-A16
pair abolishes specific affinity for HIV-RT and
modification of G16-N1 strongly interferes with binding
30   to HIV-RT. This modification of G16-N1 must prevent a
crucial contact with the protein. Why G substitutions
for U1 reduce affinity and A substitutions do not is
not clear. Admittedly the G substitution is in a
context in which the 5' end of the RNA is one
35   nucleotide shorter, however synthetic RNAs in which U1
is the 5' terminal nucleotide bind with unchanged
affinity from those _in vitro_ transcripts with two extra

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Gs at the 5' end (Figure 7). Perhaps A at U1 replaces
a potential U interaction with a similar or different
interaction with HIV-RT a replacement that cannot be
performed by C or G at this position.

5   The next base-pair of Stem 1 (C2-G15) cannot be
replaced by a G-C base-pair without complete loss of
specific affinity for HIV-RT. Modification of the
base-pairing faces of either nucleotide strongly
interferes with binding to HIV-RT and binding with

10   HIV-RT protects from these modifications. Substitution
of the next base-pair, C3-G14, with a G-C pair shows
less drastic reduction of affinity, but modification is
strongly interfering at this position. Substitution of
a C-G pair for G4-C13 has no effect on binding, and

15   substitution of the less stable A-U and U-A pairs allow
some specific affinity. Substitution of the
non-pairing A-C for these positions abolishes specific
binding. This correlates with the appearance of C-G
substitutions and one A-U substitution in the original

20   SELEX phylogeny at this position, the non-reactivity of
this base-pair in the native state, and the high degree
of modification interference found for these bases.
      The chemical modification data of Loop 2
corroborate well the phylogenetic conservation seen in

25   the original SELEX experiments. Strong modification
interference is seen at positions A17 and A19. Weak
modification interference occurs at A20 which
correlates with the finding of some Loop 2's of the
original SELEX that are deleted at this relative

30   position (although the chemical interference
experiments conducted do not exhaustively test all
potential contacts that a base may make with HIV-RT).
A18 is unconserved in the original SELEX and
modification at this position does not interfere, nor

35   is this position protected from modification by binding
to HIV-RT.
      Taken together the above data suggest that the

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essential components of Stem 1 are a single-stranded 5'
nucleotide (U or A) which may make sequence specific
contact with the protein and a three base-pair helix
(C2-G15, C3-G14, G4-C13) where there are

5   sequence-specific interactions with the HIV-RT at the
first two base-pairs and a preference for a strong
base-pair (i.e. either C-G or G-C) at the third loop
closing position of G4-C13. Loop 2 should be more
broadly described as GAXAA (16-20) due to the

10   single-stranded character of G16 which probably
interacts with HIV-RT in a sequence-specific manner, as
likely do A17 and A19. Stem 2 varies considerably in
the pattern and number of base-pairing nucleotides, but
from 3' deletion experiments reported here one could

15   hypothesize that a minimum of 3 base-pairs in Stem 2
are required for maximal affinity. Within the context
of eight nucleotides connecting the two strands
comprising the helix of Stem 1, at least 2 nucleotides
are required in Loop 1 of the bound ligand.

20   The revised ligand description for HIV-RT obtained
based on the methods of this invention is shown in
Figure 11. The major differences between that shown in
Figure 1 (which is based on the original and secondary
SELEX consensuses) is the length of Stem 2, the more

25   degenerate specification of the base-pair G4-C13, the
size of Loop 1 (which is directly related to the size
of Stem 2) and the single-stranded character of U1 and
G16.

How can these differences be reconciled? Although

30   not limited by theory, the SELEX strategy requires 5'
and 3' fixed sequences for replication. In any RNA
sequence, such additional sequences increase the
potential for other conformations that compete with
that of the high-affinity ligand. As a result,

35   additional structural elements that do not directly
contribute to affinity, such as a lengthened Stem 2,
may be selected. Given that the first two base pairs

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of Stem 1 must be C-G because of sequence-specific
contacts the most stable closing base-pair would be
G4-C13 (Freier et al. (1986) Proc. Natl. Acad. Sci.
USA. 83:9373) again selected to avoid conformational
5   ambiguity. The sequence-specific selection of U1 and
G16 may be coincidental to their ability to base-pair;
in other nucleic acid ligand-protein complexes such as
Klenow fragment/primer-template junction and tRNA/tRNA
synthetase there is significant local denaturation of
10   base-paired nucleotides (Freemont et al. (1988) Proc.
Natl. Acad. Sci. USA 85:8924; Ronald et al. (1989)
Science 246:1135) which may also occur in this case.

VI.   Performance of Walking Experiment with HIV-RT
15   Nucleic Acid Ligand to Identify Extended Nucleic
Acid Ligands.
It had previously been found that fixed sequences
(of 28 nucleotides) placed 5' to the pseudoknot
consensus ligand reduced the affinity to HIV-RT and
20   that sequences (of 31 nucleotides) added 3' to the
ligand increased that affinity. A SELEX experiment was
therefore performed in which a 30 nucleotide variable
region was added 3' to the ligand B sequence to see if
a consensus of higher affinity ligands against HIV-RT
25   could be obtained. Individual isolates were cloned and
sequenced after the sixteenth round. The sequences are
listed in Figure 9 grouped in two motifs (SEQ ID
NO:115-135). A schematic diagram of the secondary
structure and primary sequence conservation of each
30   motif is shown in Figure 10. The distance between the
RNase H and polymerase catalytic domains of HIV-RT has
recently been determined to be on the order of 18
base-pairs of an A-form RNA-DNA hybrid docked (by
computer) in the pocket of a 3.5 Å resolution structure
35   derived from X-ray crystallography (Kohlstaedt et al.
(1992) Science 256:1783). The distance from the
cluster of bases determined to be crucial to this

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interaction in the pseudoknot and the conserved bases
in the extended ligand sequence is approximately 18
base-pairs as well. Accordingly, it is concluded that
the pseudoknot interacts with the polymerase catalytic
site -- in that the ligand has been shown to bind HIV-
RT deleted for the RNAse H domain -- and that the
evolved extension to the pseudoknot may interact with
the RNAse H domain. In general the ligands tested from
each of these motifs increase affinity of the ligand B
sequence to HIV-RT by at least 10-fold.

VII. ELUCIDATION OF AN IMPROVED NUCLEIC ACID LIGAND FOR
     HIV-1 REV PROTEIN.
         An example of the methods of the present invention
are presented herein for the nucleic acid ligand for
HIV-1 Rev protein. U.S. Patent Application Serial No.
07/714,131 and PCT Patent Application Publication
WO91/19813 describe the results obtained when SELEX was
performed with the Rev target. Inspection of the
nucleic acid sequences that were found to have a high
affinity to Rev revealed a grouping of these sequences
into three Motifs (I,II, and III). Ligands of Motif I
seemed to be a composite of the individual motifs
described by Motifs II and III, and in general bound
with higher affinity to Rev. One of the Motif I ligand
sequences (Rev ligand sequence 6a) bound with
significantly higher affinity than all of the ligands
that were cloned and sequenced. As shown in Figure 12,
the 6a sequence is hypothesized to form a bulge between
two helices with some base-pairing across this bulge.
         Described herein are chemical modification
experiments performed on ligand 6a designed to confirm
the proposed secondary structure, find where binding of
the Rev protein protects the ligand from chemical
attack, and detect the nucleotides essential for Rev
interaction. In addition, a secondary SELEX experiment
was conducted with biased randomization of the 6a

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ligand sequence so as to more comprehensively describe
a consensus for the highest affinity binding to the
HIV-1 Rev protein.

Chemical modification of the Rev ligand. Chemical
5   modification studies of the Rev ligand 6a were
undertaken to determine its possible secondary
structural elements, to find which modifications
interfere with the binding of the ligand by Rev, to
identify which positions are protected from
10   modification upon protein binding, and to detect
possible changes in ligand structure that occur upon
binding.

The modifying chemicals include ethylnitrosourea
(ENU) which modifies phosphates, dimethyl sulfate (DMS)
15   which modifies the base-pairing positions N3 of C and
N1 of adenine, kethoxal which modifies base-pairing
positions N1 and N2 of guanine, carbodiimide (CMCT)
which modifies base-paring position N3 of uracil and to
a smaller extent the N1 position of guanine, and
20   diethylpyrocarbonate (DEPC) which modifies the N7
position of adenine and to some extent also the N7 of
guanine. ENU modification was assayed by modification-
dependent hydrolysis of a labeled RNA chain, while all
other modifying agents were used on an extended RNA
25   ligand, with modified positions revealed by primer
extension of an annealed oligonucleotide.

The chemical probing of the Rev ligand native
structure is summarized in Figure 13. The computer
predicted secondary structure (Zuker (1989) supra;
30   Jaeger et al. (1989), Proc. Natl. Acad. Sci. USA
86:7706) and native modification data are in general
agreement; the ligand is composed of three helical
regions, one four-base hairpin loop, and three "bulge"
regions (see Figure 13 for a definition of these
35   structural "elements").

ENU modification of phosphates was unchanged for
ligands under native and denaturing conditions,

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indicating no involvement of phosphate groups in the
secondary or tertiary structure of the RNA. In
general, all computer-predicted base-pairing regions
are protected from modification. One exception is the
5   slight modifications of N7 (G^{10}, A^{11}, G^{12}) in the central
helix (normally a protected position in helices).
These modifications are possibly a result of helical
breathing; the absence of base-pairing face
modifications in the central helix suggest that the N7
10   accessibility is due to small helical distortions
rather than a complete, local unfolding of the RNA.
The G19-U22 hairpin loop is fully modified, except for
somewhat partial modification of G19.

The most interesting regions in the native
15   structure are the three "bulge" regions, U8-U9, A13-
A14-A15, and G26-A27. U8-U9 are fully modified by
CMCT, possibly indicating base orientations into
solvent. A13, A14, and A15 are all modified by DMS and
DEPC with the strongest modifications occurring on the
20   central A14. The bulge opposite to the A13-A15 region
shows complete protection of G26 and very slight
modification of A27 by DMS. One other investigation of
Rev-binding RNAs (Bartel et al. (1991) Cell 67:529) has
argued for the existence of A:A and A:G non canonical
25   base pairing, corresponding in the present ligand to
A13:A27 and A15:G26. These possibilities are not ruled
out by this modification data, although the isosteric
A:A base pair suggested by Bartel et al. would use the
N1A positions for base-pairing and would thus be
30   resistant to DMS treatment. Also, an A:G pair would
likely use either a N1A or N7A for pairing, leaving the
A resistant to DMS or DEPC.

Modification interference of Rev binding. The
results of the modification interference studies is
35   summarized in Figure 14 (quantitative data on
individual modifying agents is presented in Figures 15
through 19). In general, phosphate and base

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modification binding interference is clustered into two
regions of the RNA ligand. To a first approximation,
these regions correspond to two separate motifs present
in the SELEX experiments that preceded this present
5   study. Phosphate modification interference is probably
the most suggestive of actual sites for ligand-protein
contacts, and constitutes an additional criterion for
the grouping of the modification interference data into
regions.

10   The first region is centered on U24-G25-G26, and
includes interference due to phosphate, base-pairing
face, and N7 modifications. These same three
nucleotides, conserved in the wild-type RRE, were also
found to be critical for Rev binding in a modification
15   interference study using short RNAs containing the RRE
IIB stem loop (Kjems et al. (1992) EMBO J. 11:1119).
The second region centers around G10-A11-G12 with
interference again from phosphate, base-pairing face,
and N7 modifications. Additionally, there is a smaller
20   "mini-region" encompassing the stretch C6-A7-U8, with
phosphate and base-pairing face modifications
interfering with binding.

Throughout the ligand, many base-pairing face
modifications showed binding interference, most likely
25   because of perturbations in the ligand's secondary
structure. Two of the "bulge" bases, U9 and A14, did
not exhibit modification interference, indicating that
both have neither a role in specific base-pairing
interactions/stacking nor in contacting the protein.

30   Chemical modification protection when RNA is bound
to Rev. The "footprinting" chemical modification data
is summarized in Figure 20. Four positions, U8, A13,
A15, and A27, showed at least two-fold reduction in
modification of base-pairing faces (and a like
35   reduction in N7 modification for the A positions) while
bound to Rev protein. The slight N7 modifications of
G10-A11-G12 under native conditions were not detected

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when the ligand was modified in the presence of Rev.
G32, unmodified in chemical probing of the RNA native
structure, shows strong modification of its base-
pairing face and the N7 position when complexed with
5   Rev. U31 and U33, 5' and 3' of G32, show slight CMCT
modification when the ligand is bound to protein.

Secondary SELEX using biased randomization of
template. A template was synthesized as shown in Figure
21 in which the Rev ligand 6a sequence was mixed with
10   the other three nucleotides at each position in the
ratio of 62.5 (for the 6a sequence) to 12.5 for each of
the other three nucleotides. This biased template gave
rise to RNAs with background affinity for Rev protein
($Kd = 10^{-7}$). Six rounds of SELEX yielded the list of
15   sequences shown in Figure 21. The frequency
distribution of the nucleotides and base pairs found at
each position as it differs from that expected from the
input distribution during template synthesis is shown
in Figures 22 and 23. A new consensus based on these
20   data is shown in Figure 24. The most significant
differences from the sequence of Rev ligand 6a are
replacement of the relatively weak base pair A7-U31
with a G-C pair and allowed or prefered substitution of
U9 with C, A14 with U, U22 with G. Absolutely
25   conserved positions are at sites G10, A11, G12; A15,
C16, A17; U24, G25; and C28, U29, C30. No bases were
found substituted for G26 and A25, although there was
one and three deletions found at those positions
respectively.   Two labeled transcripts were
30   synthesized, one with a simple ligand 6a-like sequence,
and one with substitutions by the significant
preferences found in Figure 24. These RNAs bound
identically to Rev protein.

Most of the substitutions in the stem region
35   increase its stability. There does not seem to be
significant selection of stems of length longer than 5
base-pairs although this could be a selection for

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replicability (for ease of replication during the
reverse transcription step of SELEX, for example).
There is some scattered substitution of other
nucleotides for U9 in the original SELEX reported in
5   U.S. patent application serial number 07/714,131 filed
June 10, 1991 and PCT Patent Application Publication WO
91/19813 published December 26, 1991, but this
experiment shows prefered substitution with C.
Deletions of A27 also appeared in that original SELEX.
10   A surprising result is the appearance of C18-A pairings
in place of C18-G23 at a high frequency.

        The reason there may be preferences found in this
experiment that do not improve measured binding
affinity may lie in the differences in the binding
15   reactions of SELEX and these binding assays. In SELEX
a relatively concentrated pool of heterogeneous RNA
sequences (flanked by the requisite fixed sequences)
are bound to the protein. In binding assays low
concentrations of homogeneous RNA sequence are bound.
20   In SELEX there may be selection for more discriminating
conformational certainty due to the increased
probability of intermolecular and intramolecular
contacts with other RNA sequences. In the therapeutic
delivery of concentrated doses of RNA ligands and their
25   modified homologs, these preferences found in secondary
SELEXes may be relevant.

        Nucleic Acid Ligands to the HIV-1 tat Protein.
The present invention applies the SELEX procedure to a
specific target, the HIV-1 tat protein. In Example III
30   below, the experimental parameters used to isolate and
identify the nucleic acid ligand solution to the HIV-1
tat protein are described. Figure 26 lists the nucleic
acids that were sequenced after 10 iterations of the
SELEX process.

35   Figure 25 shows the naturally occurring TAR
sequence that has been found to be a natural ligand to
the tat protein. The specific site of interaction

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between the tat protein and the TAR sequence has been
determined, and is also identified in Figure 25.

The sequences presented in Figure 26 are grouped
into three "motifs". Each of these motifs represents a
5   nucleic acid ligand solution to the HIV-1 tat protein.
Regions of primary sequence conservation within each
motif are boxed with dashed lines. Motifs I and II
contain a common structure that places conserved
sequences (those sequences found in all or most all of
10 the nucleic acid sequences that make up the given
motif) in a bulge flanked by helical elements. The
primary sequence conservation -- which is mainly in the
single stranded domains of each bulge -- are also
similar between motifs I and II. The third motif (III)
15 is characterized by a large loop. The three motifs are
depicted schematically in Figure 27. There is no
apparent similarity between the nucleic acid ligands
identified herein and the TAR sequence given in Figure
25.

20   A boundary analysis determination was performed on
one of the ligand sequences in motif III. The
boundaries of recognition are indicated by a solid-
lined box in Figure 26. The boundary determination was
performed according to previously described techniques.
25 See, Tuerk et al. (1990) J. Mol. Biol. 213:749; Tuerk &
Gold (1990) Science 249:505.

In Figure 28, the binding affinities of sequences
7 (motif I), 24 (motif II), 29 (motif II), 31 (motif
III) and the original candidate mixture are depicted.
30 As can be seen, members from each of the nucleic acid
ligand solution motifs have increased affinity to the
tat protein relative to the candidate mixture of
nucleic acids. Each of the ligands exhibits a
significantly greater affinity to the tat protein
35 relative to the TAR sequence.

In order to produce nucleic acids desirable for
use as a pharmaceutical, it is preferred that the

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nucleic acid ligand 1) binds to the target in a manner
capable of achieving the desired effect on the target;
2) be as small as possible to obtain the desired
effect; 3) be as stable as possible; and 4) be a
5   specific ligand to the chosen target. In most, if not
all, situations it is preferred that the nucleic acid
ligand have the highest possible affinity to the
target.

        This invention includes the specific nucleic acid
10   ligands shown in Figure 26 and the nucleic acid ligand
solutions as depicted schematically in Figure 27. The
scope of the ligands covered by this invention extends
to all ligands to the tat protein identified according
to the SELEX procedure. More specifically, this
15   invention includes nucleic acid sequences that are 1)
substantially homologous to and that have substantially
the same ability to bind the tat protein as the
specific nucleic acid ligands shown in Figure 26 or
that are 2) substantially homologous and that have
20   substantially the same ability to bind the tat protein
as the nucleic acid ligand solutions shown in Figure
27. By substantially homologous, it is meant, a degree
of primary sequence homology in excess of 70%, most
preferably in excess of 80%. Substantially the same
25   ability to bind the tat protein means that the affinity
is within two orders of magnitude of the affinity of
the substantially homologous sequence described herein.
It is well within the skill of those of ordinary skill
in the art to determine whether a given sequence --
30   substantially homologous to those specifically
described herein -- has substantially the same ability
to bind the tat protein.

        A review of motifs I, II and III, and the binding
curves shown in Figure 28, show that sequences that
35   have little or no primary sequence homology may still
have substantially the same ability to bind the tat
protein. If one assumes that each of these motifs of

ligands binds the same binding site of the _tat_ protein,
it is clear that binding is controlled by the secondary
or tertiary structure of the nucleic acid ligand.
Certain primary structures -- represented by motifs I,
II and III herein -- are apparently able to assume
structures that appear very similar to the binding site
of the _tat_ protein. For these reasons, the present
application also includes nucleic acid ligands that
have substantially the same structural form as the
ligands presented herein and that have substantially
the same ability to bind the _tat_ protein as the nucleic
acid ligands shown in Figure 26 or Figure 27. Wherein
substantially the same structure includes all nucleic
acid ligands having the common structural elements of
motifs I, II and III that lead to the affinity to the
tat protein.

        This invention also includes the ligands as
described above, wherein certain chemical modifications
have been made in order to increase the _in vivo_
stability of the ligand or to enhance or mediate the
delivery of the ligand.

        The nucleic acid ligands and nucleic acid ligand
solutions to the HIV-1 _tat_ protein described herein are
useful as pharmaceuticals and as part of gene therapy
treatments. According to methods known to those
skilled in the art, the nucleic acid ligands may be
introduced intracellularly into cells infected with the
HIV virus, where the nucleic acid ligand will compete
with the TAR sequence for the _tat_ protein. As such,
transcription of HIV genes can be prevented.

        Nucleic Acid Ligands to Thrombin. This invention
includes the specific nucleic acid ligands shown in
Figure 29 (SEQ ID NO:137-155). The scope of the
ligands covered by this invention extends to all
ligands to thrombin identified according to the SELEX
procedure. More specifically, this invention includes
nucleic acid sequences that are substantially

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homologous to and that have substantially the same
ability to bind thrombin as the specific nucleic acid
ligands shown in Figure 29 (SEQ ID NO:137-155).

5   A review of the proposed structural formations
shown in Figure 30 for the group I and group II ligands
shows that sequences that have little or no primary
sequence homology may still have substantially the same
ability to bind thrombin. For these reasons, the
present invention also includes RNA ligands that have
10   substantially the same structure as the ligands
presented herein and that have substantially the same
ability to bind thrombin as the RNA ligands shown in
Figure 30 (SEQ ID NO:156-159). "Substantially the same
structure" includes all RNA ligands having the common
15   structural elements of the sequences given in Figure 30
(SEQ ID NO:156-159).

          This invention also includes the ligands as
described above, wherein certain chemical modifications
have been made in order to increase the _in vivo_
20   stability of the ligand or to enhance or mediate the
delivery of the ligand. Specifically included within
the scope of this invention are RNA ligands of thrombin
that contain 2'-NH$_2$ modifications of certain riboses of
the RNA ligand.

25   The nucleic acid ligands and nucleic acid ligand
solutions to thrombin described herein are useful as
pharmaceuticals and as part of gene therapy treatments.

          The concepts of vascular injury and thrombosis are
important in the understanding of the pathogenesis of
30   various vascular diseases, including the initiation and
progression of atherosclerosis, the acute coronary
syndromes, vein graft disease, and restenosis following
coronary angioplasty.

          The high-affinity thrombin binding RNA ligands of
35   this invention may be expected to have various
properties. These characteristics can be thought about
within the context of the hirudin peptide inhibitors

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and the current understanding of thrombin structure and
binding. Within this context and not being limited by
theory, it is most likely that the RNA ligands are
binding the highly basic anionic exosite. It is also
5   likely that the RNA is not binding the catalytic site
which has high specificity for the cationic arginine
residue. One would expect the RNA ligands to behave in
the same manner as the C-terminal Hirudin peptides. As
such, they would not strongly inhibit small peptidyl
10   substrates, but would inhibit fibrinogen-clotting,
protein C activation, platelet activation, and
endothelial cell activation. Given that within the
anionic exosite the fibrinogen-clotting and TM-binding
activities are separable, it is possible that different
15   high-affinity RNA ligands may inhibit these activities
differentially. Moreover, one may select for one
activity over another in order to generate a more
potent anticoagulant than procoagulant.
     The SELEX process for identifying ligands to a
20   target was performed using human thrombin as the
target, and a candidate mixture containing 76
nucleotide RNAs with a 30 nucleotide region of
randomized sequences (Example IV). Following twelve
rounds of SELEX, a number of the selected ligands were
25   sequenced, to reveal the existence of two groups of
sequences that had common elements of primary sequence.
     A dramatic shift in binding of the RNA population
was observed after 12 rounds of SELEX, when compared to
the bulk 30N RNA. Sequencing of bulk RNA after 12
30   rounds also showed a non-random sequence profile. The
RNA was reverse transcribed, amplified, cloned and the
sequences of 28 individual molecules were determined
(Figure 29). Based on primary sequence homology, 22 of
the RNAs were grouped as class I and 6 RNAs were
35   grouped as class II. Of the 22 sequences in class I,
16 (8 of which were identical) contained an identical
sequence motif GGAUCGAAG(N)$_2$AGUAGGC (SEQ ID NO:9),

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whereas the remaining 6 contained 1 or 2 nucleotide
changes in the defined region or some variation in N=2
to N=5. This conserved motif varied in its position
within the 30N region. In class II, 3 of the 6 RNAs

5   were identical and all of them contained the conserved
motif GCGGCUUUGGGCGCCGUGCUU (SEQ ID NO:10), beginning
at the 3rd nucleotide from the end of the 5' fixed
region.

         Three sequence variant RNA ligands from class I

10   (6, 16, and 18) and one (27) from class II, identified
by the order they were sequenced, were used for
individual binding analysis. Class I RNAs were
exemplified by clone 16 with a kD of approximately 30
^nM and the kD for the class II RNA clone 27 was

15   approximately 60 ^nM.

         In order to identify the minimal sequence
requirements for specific high affinity binding of the
76 nucleotide RNA which includes the variable 30N
region flanked by 5' and 3' fixed sequence, 5' and 3'

20   boundary experiments were performed. For 5' boundary
experiments the RNAs were 3' end labeled and hydrolyzed
to give a pool of RNAs with varying 5' ends. For the
3' boundary experiments, the RNAs were 5' end-labeled
and hydrolyzed to give a pool of RNAs with varying 3'

25   ends. Minimal RNA sequence requirements were
determined following RNA protein binding to
nitrocellulose filters and identification of labeled
RNA by gel electrophoresis.

         3' boundary experiments gave the boundaries for

30   each of the 4 sequences shown in Figure 30A (SEQ ID
NO:156-159). These boundaries were consistent at all
protein concentrations. 5' boundary experiments gave
the boundaries shown in Figure 31 plus or minus 1
nucleotide, except for RNA 16 which gave a greater

35   boundary with lower protein concentrations. Based on
these boundary experiments, possible secondary
structures of the thrombin ligands are shown in Figure

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30B.

RNAs corresponding to the smallest and largest
hairpin of class I clone 16 (24 and 39 nucleotides) and
the hairpin of class II clone 27 (33 nucleotides) were
5   synthesized or transcribed for binding analysis (see
Figure 30B). Results show that the RNA 27 hairpin
binds with affinity (kD of about 60 ^{n}M) equal to that
of the entire 72 nucleotide transcript with fixed and
variable region (compare RNA 27 in Fig. 31A with RNA
10   33R in Fig. 31C). The kDs for class I clone 16 RNA
hairpins on the other hand increased an order of
magnitude from 30 ^{n}M to 200 ^{n}M.

Modifications in the $2NH_2$-ribose of pyrimidine
residues of RNA molecules has been shown to increase
15   stability of RNA (resistant to degradation by RNase) in
serum by at least 1000 fold. Binding experiments with
the $2NH_2$-CTP/UTP modified RNAs of class I and class II
showed a significant drop in binding when compared to
the unmodified RNA (Figure 32). Binding by the bulk
20   30N RNA, however, showed a slight increase in affinity
when it was modified.

A ssDNA molecule with a 15 nucleotide consensus
5′-GGTTGGTGTGGTTGG-3′ (G15D) (SEQ ID NO:1) has been
shown to bind human thrombin and inhibit fibrin-clot
25   formation in vitro (Bock et al. (1992) supra). The
results of competition experiments for binding thrombin
between G15D and the RNA hairpin ligands of this
invention are shown in Figure 33. In the first of
these experiments A), ^{32}P-labeled G15D used as the
30   tracer with increasing concentrations of unlabeled RNA
or unlabeled G15D. As expected, when the G15D was used
to compete for its own binding, binding of labeled DNA
was reduced to 50% at equimolar concentrations (1 μM)
of labeled and unlabeled competitor DNA. Both the
35   class I clone 16 synthetic RNAs 24 and 39, and the
class II clone 27 synthetic RNA 33 were able to compete
for binding of G15D at this concentration. In B) the

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higher affinity class II hairpin RNA 33 (kD $\simeq$ 60 nM)
was ^{32}P-labelled and used as the tracer with increasing
concentrations of unlabelled RNA or unlabelled G15D DNA
(kD $\simeq$ 200 nM). In these experiments, the G15D was able
to compete effectively with RNA 33 at higher
concentrations than the RNA 33 competes itself (shift
of binding to the right), which is what is expected
when competing with a ligand with 3-4 fold higher
affinity. The class II hairpin RNA 33 (kD $\simeq$ 60 nM) was
competed only weakly by the class I hairpin RNA 24 (kD
$\simeq$ 200 nM), suggesting that while there may be some
overlap, the RNAs of these two classes bind with high
affinity to different yet adjacent or overlapping
sites. Because both of these RNAs can compete for G15D
binding, this DNA 15mer probably binds in the region of
overlap between the class I and class II hairpins.

Cleavage of Chromogenic Substrate S2238. The
ability of thrombin to cleave the peptidyl chromogenic
substrate S2238 (H-D-Phe-Pip-Arg-pNitroaniline) (H-D-
Phe-Pip-Arg-pNA) (Kabi Pharmacia) was measured in the
presence and absence of the RNA ligands of this
invention. There was no inhibitory effect of RNA on
this cleavage reaction at 10^{-8} M thrombin and 10^{-8} M
RNA, 10^{-9} M thrombin and 10^{-8} M RNA or at 10^{-8} M thrombin
and 10^{-7} M RNA (Figure 34A). These results suggest
that the RNA ligands do not bind in the catalytic site
of the enzyme.

Cleavage of Fibrinogen to Fibrin and Clot
Formation. The ability of thrombin to catalyze clot
formation by cleavage of fibrinogen to fibrin was
measured in the presence and absence of RNA. When RNA
was present at a concentration equal to the Kd (30 nM
for class I RNAs and 60 nM for class II RNAs), which
was in 5 to 10-fold excess of thrombin, clotting time
was increased by 1.5-fold (Figure 34B).

Specificity of thrombin binding. Representative
ligands from class I and class II showed that these

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ligands had low affinity for ATIII at concentrations as high as 1 μM (Figure 35A). These ligands showed reduced affinity when compared with the bulk 30N3 RNA suggesting that there has been selection against non-
5   specific binding. This is of particular importance because ATIII is an abundant plasma protein with high affinity for heparin, a polyanionic macromolecule. These results show that the evolution of a discreet structure present in the class I and class II RNAs is
10   specific for thrombin binding and, despite its polyanionic composition, does not bind to a high affinity heparin binding protein. It is also important to note that these thrombin specific RNA ligands have no affinity for prothrombin (Figure 35B), the inactive
15   biochemical precursor to active thrombin, which circulates at high levels in the plasma (≈ 1 μM).

   Nucleic Acid Ligands to Basic Fibroblast Growth
Factor (bFGF). The present invention applies the SELEX procedure to a specific target, bFGF. In the Example
20   section below, the experimental parameters used to isolate and identify the nucleic acid ligand solutions to bFGF are described.

   This invention includes the specific nucleic acid ligands shown in Tables II-IV. The scope of the
25   ligands covered by this invention extends to all ligands to bFGF identified according to the SELEX procedure. More specifically, this invention includes nucleic acid sequences that are substantially homologous to and that have substantially the same
30   ability to bind bFGF as the specific nucleic acid ligands shown in Tables II-IV.

   A review of the proposed structural formations shown in Figure 41 for the family 1 and 2 ligands shows that sequences that have little or no primary sequence
35   homology may still have substantially the same ability to bind bFGF. The present invention also includes RNA ligands that have substantially the same structure as

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the ligands presented herein and that have substantially the same ability to bind bFGF as the RNA ligands shown in Tables II and III. "Substantially the same structure" includes all RNA ligands having the

5   common structural elements of the sequences given in Tables II and III (SEQ ID NO:27-67).

This invention also includes the ligands described above, wherein certain chemical modifications have been made in order to increase the _in vivo_ stability of the

10   ligand, enhance or mediate the delivery of the ligand, or reduce the clearance rate from the body. Specifically included within the scope of this invention are RNA ligands of bFGF that contain $2'-NH_2$ modifications of certain riboses of the RNA ligand.

15   The nucleic acid ligands and nucleic acid ligand solutions to bFGF described herein are useful as pharmaceuticals, and as part of gene therapy treatments. Further, the nucleic acid ligands to bFGF described herein may be used beneficially for

20   diagnostic purposes.

The high-affinity nucleic acid ligands of the present invention may also have various properties, including the ability to inhibit the biological activity of bFGF. Representative ligands from sequence

25   family 1 and 2 were found to inhibit binding of bFGF to both low- and high-affinity cell-surface receptors. These nucleic acid ligands may be useful as specific and potent neutralizers of bFGF activity _in vivo_.

30   EXAMPLE I: ELUCIDATION OF IMPROVED NUCLEIC ACID LIGAND SOLUTION FOR HIV-RT

RNA synthesis. In vitro transcription with oligonucleotide templates was conducted as described by Milligan et al. (1987) supra. All synthetic nucleic

35   acids were made on an Applied Biosystems model 394-08 DNA/RNA synthesizer using standard protocols. Deoxyribonucleotide phosphoramidites and DNA synthesis

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solvents and reagents were purchased from Applied
Biosystems. Ribonucleotide and
2'-methoxy-ribonucleotide phosphoramidites were
purchased from Glen Research Corporation. For mixed
5   base positions, 0.1 M phosphoramidite solutions were
mixed by volume to the proportions indicated. Base
deprotection was carried out at 55°C for 6 hours in 3:1
ammonium hydroxide:ethanol. t-butyl-dimethylsilyl
protecting groups were removed from the 2'-OH groups of
10   synthetic RNAs by overnight treatment in
tetrabutylammonium fluoride. The deprotected RNAs were
then phenol extracted, ethanol precipitated and
purified by gel electrophoresis.

Affinity assays with labeled RNA and HIV-RT.
15   Model RNAs for refinement of the 5' and 3' boundaries
and for determination of the effect of substitutions
were labeled during transcription with T7 RNA
polymerase as described in Tuerk & Gold (1990) supra
except that a-^{32}P-ATP was used, in reactions of 0.5 mM
20   C,G, and UTP with 0.05 mM ATP. Synthetic
oligonucleotides and phosphatased transcripts (as in
Tuerk & Gold (1990) supra were kinased as described in
Gauss et al. (1987) Mol. Gen. Genet. 206:24. All
RNA-protein binding reactions were done in a "binding
25   buffer" of 200 mM KOAc, 50 mM Tris-HCl pH 7.7, 10 mM
dithiothreitol with exceptions noted for chemical
protection experiments below. RNA and protein
dilutions were mixed and stored on ice for 30 minutes
then transferred to 37°C for 5 minutes. In binding
30   assays the reaction volume was 60 ul of which 50 ul was
assayed. Each reaction was suctioned through a pre-wet
(with binding buffer) nitrocellulose filter and rinsed
with 3 mls of binding buffer after which it was dried
and counted for assays or subjected to elution and
35   assayed for chemical modification. In comparisons of
binding affinity, results were plotted and the protein
concentration at which half-maximal binding occurred

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(the approximate Kd in conditions of protein excess)
was determined graphically.

Selection of modified RNAs by HIV-RT. Binding
reactions were as above except that rather than to vary
the amount of HIV-RT added to a reaction, the volume of
reaction was increased in order to lower concentration.
RNAs that were modified under denaturing conditions
were selected at concentrations of 20, 4 and 0.8
nanomolar HIV-RT (in volumes of 1, 5 and 25 mls of
binding buffer.) The amount of RNA added to each
reaction was equivalent for each experiment
(approximately 1-5 picomoles). RNA was eluted from
filters as described in Tuerk & Gold (1990) supra) and
assayed for modified positions. In each experiment a
control was included in which unselected RNA was
spotted on a filter, eluted and assayed for modified
positions in parallel with the selected RNAs.
Determinations of variation in chemical modification
for selected versus unselected RNAs were made by visual
inspection of exposed films of electrophoresed assay
products with the following exceptions. The extent of
modification interference by ENU was determined by
densitometric scanning of films using an LKB laser
densitomer. An index of modification interference
(M.I.) at each position was calculated as follows:

M.I. = (O.D.unselected/ O.D.unselected
A20)/(O.D.selected/O.D.selected A20)

where the value at each position assayed for selected
modified RNA (O.D.selected) is divided by that value
for position A20 (O.D.selected A20) and divided into
likewise normalized values for the unselected lane.
All values of M.I. greater than 2.0 are reported as
interfering and greater than 4.0 as strongly
interfering. In determination of the effects of mixed
substitution of 2'-methoxys for 2' hydroxyls (on the

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ribose at each nucleotide position) gels of
electrophoresed hydrolysis products were counted on an
Ambis detection system directly. The counts associated
with each band within a lane were normalized as shown
above but for position A17. In addition,
determinations were done by laser densitometry as
described below.

Chemical modification of RNA. A useful review of
the types of chemical modifications of RNA and their
specificities and methods of assay was done by
Ehresmann et al. (1987) supra. Modification of RNA
under native conditions was done at 200 mM KOAc, 50 mM
Tris-HCl pH 7.7 at 37°C with ethylnitrosourea (ENU)
(1/5 dilution v/v of room temperature ENU-saturated
ethanol) for 1-3 hours, dimethyl sulfate (DMS)
(1/750-fold dilution v/v) for eight minutes, kethoxal
(0.5 mg/ml) for eight minutes, carbodiimide (CMCT) (8
mg/ml) for 20 minutes, and diethyl pyrocarbonate (DEPC)
(1/10 dilution v/v for native conditions or 1/100
dilution for denaturing conditions) for 45 minutes, and
under the same conditions bound to HIV-RT with the
addition of 1 mM DTT. The concentrations of modifying
chemical reagent were identical for denaturing
conditions (except where noted for DEPC); those
conditions were 7M urea, 50 mM Tris-HCl pH 7.7, 1 mM
EDTA at 90°C for 1-5 minutes except during modification
with ENU which was done in the absence of 7M urea.

Assay of chemical modification. Positions of
chemical modification were assayed by reverse
transcription for DMS, kethoxal and CMCT on the
lengthened ligand B RNA,
5'-GGUCCGAAGUGCAACGGGAAAAUGCACUAUGAAAGAAU-UUUAUAUCUCUAU
UGAAAC-3' (SEQ ID NO:11) (the ligand B sequence is
underlined), to which is annealed the oligonucleotide
primer 5'-CCGGATCCGTTTCAATAGAG-ATATAAAATTC-3'(SEQ ID
NO:12); reverse transcription products (obtained as in
Gauss et al., 1987 supra) were separated by

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electrophoresis on 10% polyacrylamide gels. Positions
of ENU and DEPC modification were assayed as in Vlassov
et al. (1980) FEBS Lett. 120:12 and Peattie and Gilbert
(1980) Proc. Natl. Acad. Sci. USA 77:4679, respectively
5   (separated by electrophoresis on 20% polyacrylamide
gels). Assay of 2'-methoxy ribose versus ribose at
various positions was assayed by alkaline hydrolysis
for 45 minutes at 90°C in 50 mM sodium carbonate pH
9.0.

10   Modification of RNA in the presence of HIV-RT.
Conditions were as for modification of native RNA.
Concentrations of HIV-RT were approximately 10-fold
excess over RNA concentration. In general protein
concentrations ranged from 50 nM to 1 uM.

15   SELEX isolation of accessory contacts with HIV-RT.
The starting RNA was transcribed from PCRd templates
synthesized from the following oligonucleotides:

5'-GGGCAAGCTTTAATACGACTCACTATAGGTCCGAAGTGCAACGGGAAAATG-
20   CACT-3' (5' primer) (SEQ ID NO:13),

5'-GTTTCAATAGAGATATAAAATTCTTTCATAG-3' (3' primer) (SEQ
ID NO:14),

25   5'-GTTTCAATAGAGATATAAAATTCTTTCATAG-[30N]AGTGCATTTTCCCGT
TGC-ACTTCGGACC-3' (variable template)(SEQ ID NO:15).

SELEX was performed as described previously with HIV-RT
30   with the following exceptions. The concentration of
HIV-RT in the binding reaction of the first SELEX round
was 13 nanomolar, RNA at 10 micromolar, in 4 mls of
binding buffer, in the rounds 2 through 9 selection was
done with 2.6 nanomolar HIV-RT, 1.8 micromolar RNA in
35   20 mls of buffer, in rounds 10-14 we used 1 nanomolar
HIV-RT, 0.7 micromolar RNA in 50 mls, and for rounds 15
and 16 we used 0.5 nanomolar HIV-RT, 0.7 micromolar RNA
in 50 mls of binding buffer.

40   REFERENCES TO EXAMPLE I:
         Ehresman, C., Baudin, F., Mougel, M., Romby, P.,

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Ebel, J-P. Ehresman, B. (1987) Probing the structure of RNAs in solution. Nuc. Acids. Res. 15:9109-9128.

Freemont, P.S., Friedman, J.M., Beese, M.R., Sanderson, M.R. and Steitz, T.A. (1988) Proc. Natl. Acad. Sci. USA 85:8924.

Freier, S.M., Kierzed, R., Jaeger, J.A., Suigimoto, N., Caruthers, M.H., Neilson, T., and Turner, D.H. (1986) Proc. Natl. Acad. Sci. USA 83:9373-9377.

Gauss, P., Gayle, M., Winter, R.B., Gold, L. (1987) Mol. Gen. Genet. 206:24.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1992) Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256:1783-1790.

Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. 15:8783-8798.

Moazed, D., Stern, S. and Noller, H. (1986) Rapid chemical probing of conformation in 16S ribosomal RNA and 30S ribosomal subunits using primer extension. J. Mol. Biol. 187:399-416.

Peattie, D. and Gilbert, W. (1980) Chemical probes for higher order structure in RNA. Proc. Natl. Acad. Sci. USA 77:4679-4682.

Peattie, D. and Herr, W. (1981) Chemical probing of the tRNA-ribosome complex. Proc. Natl. Acad. Sci. USA 78:2273-2277.

Roald, M.A., Perona, J., Söll, D. and Steitz, T.A. (1989) Science 246:1135.

Tuerk, C., Eddy, S., Parma, D. and Gold, L. (1990) The translational operator of bacteriophage T4 DNA polymerase. J. Mol. Biol. 213:749.

Tuerk, C. and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505-510.

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Tuerk, C., MacDougal, S. and Gold, L. (1992a) RNA pseudoknots that inhibit HIV-1 reverse transcriptase. Proc. Natl. Acad. Sci. USA 89:6988-6992.

Vlassov, V., Giege, R. and Ebel, J.-P. (1980) The tertiary structure of yeast tRNAPhe in solution studied by phosphodiester bond modification with ethylnitrosourea. FEBS 120:12-16.

EXAMPLE II: ELUCIDATION OF IMPROVED NUCLEIC ACID LIGAND SOLUTIONS FOR HIV-1 REV PROTEIN

The Rev ligand sequence used for chemical modification is shown in Figure 12 (the numbering scheme shown will be used hereinafter). RNA for modification was obtained from T7 RNA polymerase transcription of synthetic oligonucleotide templates. ENU modification was carried out on the ligand sequence as shown in Figure 12. DMS, kethoxal, CMCT, and DEPC modifications were carried out on a extended ligand sequence, and analyzed by reverse transcription with the synthetic oligonucleotide primer shown in Figure 12.

Chemical Modification of RNA. Chemical modification techniques for nucleic acids are described in general in Ehresmann et al. (1987) supra. Modification of RNA under native conditions was performed in 200mM KOAc, 50mM Tris-HCl pH 7.7, 1 mM EDTA at 37°C. Modification under denaturing conditions was done in 7M urea, 50mM Tris-HCl pH 7.7 at 90°C. Concentration of modifying agents and incubation times are as follows: ethylnitrosourea (ENU)- 1/5 dilution v/v of ethanol saturated with ENU, native 1-3 hours, denaturing 5 minutes; dimethyl sulfate (DMS)- 1/750-fold dilution v/v, native 8 minutes, denaturing 1 minute; kethoxal- 0.5 mg/ml, native 5 minutes, denaturing 2 minutes; carbodiimide (CMCT)- 10 mg/ml, native 30 minutes, denaturing 3 minutes; diethyl pyrocarbonate (DEPC)- 1/10 dilution v/v, native 10

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minutes, denaturing 1 minute.

Modification interference of Rev binding. RNAs
chemically modified under denaturing conditions were
selected for Rev binding through filter partitioning.
Selections were carried out at Rev concentrations of
30, 6, and 1.2 nanomolar (in respective volumes of 1,
5, and 25 mls of binding buffer; 200 mM KOAc, 50 mM
Tris-HCl pH 7.7, and 10 mM dithiothreitol).
Approximately 3 picomoles of modified RNA were added to
each protein solution, mixed and stored on ice for 15
minutes, and then transferred to 37°C for 10 minutes.
Binding solutions were passed through pre-wet
nitrocellulose filters, and rinsed with 5 mls of
binding buffer. RNA was eluted from the filters as
described in Tuerk & Gold (1990) supra and assayed for
modified positions that remained. Modified RNA was
also spotted on filters and eluted to check for uniform
recovery of modified RNA.

The extent of modification interference was
determined by densitometric scanning of autoradiographs
using LKB (ENU) and Molecular Dynamics (DMS, kethoxal,
CMCT, and DEPC) laser densitometers. Values for
modified phosphates and bases were normalized to a
chosen modified position for both selected and
unselected lanes; the values for the modified positions
in the selected lane were then divided by the
corresponding positions in the unselected lane (for
specific normalizing positions see Figures 15-19).
Values above 4.0 for modified bases and phosphates are
designated as strongly interfering, and values above
2.0 are termed slightly interfering.

Modification of RNA in the presence of Rev.
"Footprinting" of the Rev ligand, modification of the
RNA ligand in the presence of Rev protein, was
performed in 200mM KOAc, 50mM Tris-Cl pH 7.7, 1mM DTT,
and 5mM MgCl. Concentration of protein was 500
nanomolar, and approximately in 3-fold molar excess

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over RNA concentration. Modification with protein present was attempted with all modifying agents listed above except ethylnitrosourea (ENU).

    <u>Assay of chemically modified RNA</u>. Positions of
5   ENU modification were detected as in Vlassov <u>et al</u>. (1980) <u>supra</u> and separated by electrophoresis on 20% denaturing acrylamide gels. DMS, kethoxal, CMCT, and DEPC were assayed by reverse transcription of the extended Rev ligand with a radiolabelled
10 oligonucleotide primer (Figure 12) and separated by electrophoresis on 8% denaturing acrylamide gels.

    <u>SELEX with biased randomization</u>. The templates for <u>in vitro</u> transcription were prepared by PCR from the following oligonucleotides:

15

5'-CCCGGATCCTCTTTACCTCTGTGTGagatascagagtccacaacgtg
ttctcaatgacccGGTCGGAAGGCCATCAATAGTCCC-3' (template
oligo) (SEQ ID NO:16),

20 5'-CCGAAGCTTAATACGACTCACTATAGGGACTATTGATGGGCCTTCCGACC-
3' (5' primer) (SEQ ID NO:17),

5'-CCCGGATCCTCTTTACCTCTGTGTG-3' (3' primer) (SEQ ID
NO:18)
25

where the small case letters in the template oligo indicates that at each position that a mixture of reagents were used in synthesis by an amount of 62.5%
30 of the small case letter, and 12.5% each of the other three nucleotides.

    SELEX was conducted as described previously with the following exceptions. The concentration of HIV-1 Rev protein in the binding reactions of the first and
35 second rounds was 7.2 nanomolar and the RNA 4 micromolar in a volume of 10 mls (of 200 mM potassium acetage, 50 mM Tris-HCl pH 7.7, 10 mM DTT). For rounds three through six the concentration of Rev protein was 1 nanomolar and the RNA 1 micromolar in 40 mls volume.
40 HIV-1 Rev protein was purchased from American Biotechnologies, Inc.

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EXAMPLE III: NUCLEIC ACID LIGANDS TO THE HIV-1 tat
PROTEIN.

        SELEX on HIV-1 tat Protein.   tat protein was
purchased from American Bio-Technologies, Inc.
5   Templates for in vitro transcription were produced by
PCR using the following oligonucleotides:

        5'-CCGAAGCTTAATACGACTCACTATAGGGAGCTCAGAATAAACGCTCAA-3'
            (5' primer) (SEQ ID NO:18),
10

        5'-GCCGGATCCGGGCCTCATGTCGAA-[40n]-TTGAGCGTTTATTCTGAGC
        TCCC-3' (variable template) (SEQ ID NO:19),

        5'-GCCGGATCCGGGCCTCATGTCGAA-3' (3' primer)(SEQ ID
15 NO:20),

        SELEX rounds were conducted as described in Tuerk et
al. (1992a) supra, and in the SELEX Applications, under
the following conditions: Binding reactions were done
20   with 13 nanomolar tat protein and 1.3 micromolar RNA in
a volume of 2 mls for rounds 1 and 2, and 6.5 nanomolar
tat protein and 0.65 micromolar RNA in 4 mls for rounds
3-10.

        RNA synthesis.   In vitro transcription with
25   oligonucleotide templates was conducted as described by
Milligan et al. (1987) supra. All synthetic nucleic
acids were made on the Applied Biosystems model 394-08
DNA/RNA synthesizer using standard protocols.
Deoxyribonucleotide phosphoramidites and DNA synthesis
30   solvents and reagents were purchased from Applied
Biosystems.

        Affinity assays with labeled RNA and HIV-1 tat
protein. Model RNAs for refinement of the 5' and 3'
boundaries and for determination of the effect of
35   substitutions were labeled during transcription with T7
RNA polymerase as described in the SELEX Applications,
except that α-^{32}P-ATP was used, in reactions of 0.5 mM
C, G, and UTP with 0.05 mM ATP. All RNA-protein
binding reactions were done in a "binding buffer" of
40   200 mM KOAc, 50 mM Tris-Hcl pH 7.7, 10 mM

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dithiothreitol. RNA and protein dilutions were mixed
and stored on ice for 30 minutes then transferred to
37°C for 5 minutes. In binding assays the reaction
volume was 60 ul of which 50 ul was assayed. Each
5   reaction was suctioned through a pre-wet (with binding
buffer) nitrocellulose filter and rinsed with 3 mls of
binding buffer after which it was dried and counted for
assays or subjected to elution and assayed for chemical
modification. In comparisons of binding affinity,
10   results were plotted and the protein concentration at
which half-maximal binding occurred (the approximate Kd
in conditions of protein excess) was determined
graphically. The results of the binding assays are
given in Figure 27.

15

EXAMPLE IV: NUCLEIC ACID LIGANDS TO THROMBIN
     High affinity RNA ligands for thrombin were
isolated by SELEX. Random RNA molecules used for the
initial candidate mixture were generated by in vitro
20   transcription from a 102 nucleotide double-stranded DNA
template containing a random cassette 30 nucleotides
(30N) long. A population of 10^{13} 30N DNA templates
were created by PCR, using a 5' primer containing the
T7 promoter for in vitro transcription, and restriction
25   sites in both the 5' and 3' primers for cloning.
     The RNA concentration for each round of SELEX was
approximately 2-4 X 10^{-7} M and concentrations of
thrombin (Sigma, 1000 units) went from 1.0 X 10^{-6} in
the 1st round to 4.8 X 10^{-7} in rounds 2 and 3 and 2.4 X
30   10^{-7} in rounds 4-12. The binding buffer for the RNA
and protein was 100 mM NaCl, 50 mM Tris/Cl, pH7.7, 1 mM
DTT, and 1 mM $MgCl_2$. Binding was for 5 minutes at 37°C
in a total volume of 100μl in rounds 1-7 and 200μl in
rounds 8-12. Each binding reaction was filtered
35   through a pre-wetted (with 50 mM Tris/Cl, pH7.7)
nitrocellulose filter (2.5 cm Millipore, 0.45 μM) in a
Millipore filter binding apparatus, and immediately

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rinsed with 5 ml of the same buffer. The RNA was
eluted from the filters in 400 µl phenol (equilibrated
with 0.1 M NaoAc pH5.2), 200 µl freshly prepared 7 M
urea as described (Tuerk et al. (1990) supra. The RNA
was precipitated with 20 µg tRNA, and was used as a
template for cDNA synthesis, followed by PCR and in
vitro transcription to prepare RNA for the subsequent
round. The RNA was radio-labeled with ^{32}P-ATP in
rounds 1-8 so that binding could be monitored. In
order to expedite the time for each round of SELEX, the
RNA was not labeled for rounds 9-12. RNA was
prefiltered through nitrocellulose filters (1.3 cm
Millipore, 0.45 µM) before the 3rd, 4th, 5th, 8th,
11th, and 12th rounds to eliminate selection for any
nonspecific nitrocellulose binding.

Binding curves were performed after the 5th, 8th,
and 12th rounds to estimate changes in kD of the bulk
RNA. These experiments were done in protein excess at
concentrations from 1.2 X 10^{-5} to 2.4 X 10^{-9} M at a final
RNA concentration of 2 X 10^{-9} M. The RNA for these
binding curves was labeled to high specific activity
with ^{32}P-ATP or ^{32}P-UTP. Binding to nitrocellulose
filters was as described for the rounds of SELEX,
except that the filter bound RNA was dried and counted
directly on the filters.

RNA Sequencing. Following the 12th round of
SELEX, the RNA was sequenced with reverse transcriptase
(AMV, Life Sciences, Inc.) using the ^{32}P 5' end-labeled
3' complementary PCR primer.

Cloning and Sequencing individual RNAs. RNA from
the 12th round was reverse transcribed to DNA and
amplified by PCR. Digestion at restriction enzyme
sites in the 5' and 3' fixed regions were used to
remove the 30N region which was subsequently ligated
into the complementary sites in the E. coli cloning
vector pUC18. Ligated plasmid DNA was transformed into
JM103 cells and screened by blue/white colony

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formation. Colonies containing unique sequences were
grown up and miniprep DNA was prepared. Double-
stranded plasmid DNA was used for dideoxy sequencing
with the Sequenase kit version 2.0 and ^{35}S-dATP

5   (Amersham).

       End-labeling RNA. For end-labeling, RNA
transcribed with T7 polymerase was gel purified by UV
shadowing. RNA was 5′ end-labeled by dephosphorylating
the 5′ end with alkaline phosphatase 1 unit, for 30

10   minutes at 37°C. Alkaline phosphatase activity was
destroyed by phenol:chloroform extraction. RNA was
subsequently end-labeled with γ^{32}P-ATP in a reaction
with polynucleotide kinase for 30 minutes at 37°C.

       RNA was 3′ end-labeled with (5′-^{32}P)pCp and RNA

15   ligase, for 30 minutes at 37°C. 5′ and 3′ end-labeled
RNAs were gel band purified on an 8%, 8 M urea,
polyacrylamide gel.

       Determination of 5′ and 3′ boundaries. 2 pmole
RNA 3′ or 5′ end-labeled for the 5′ or 3′ boundary

20   experiments, respectively were hydrolyzed in 50 mM
Na$_2$CO$_3$ (pH 9.0) and 1 mM EDTA in a 10 μl reaction for
10 minutes at 90°C. The reaction was stopped by adding
1/5 volume 3 M NaOAc (pH5.2), and freezing at -20°C.
Binding reactions were done at 3 protein

25   concentrations, 40 nM, 10 nM and 2.5 nM, in 3 volumes
(100 μl, 400 μl, and 1600 μl, such that the amount of
protein was kept constant) containing 1X binding buffer
and 2 pmoles RNA. Reactions were incubated for 10
minutes at 37°C, filtered through a pre-wet

30   nitrocellulose membrane, and rinsed with 5 ml wash
buffer. The RNA was eluted from the filters by dicing
the filter and shaking it in 200 μl 7 M urea and 400 μl
phenol (pH 8.0) for 15 minutes at 20°C. After adding
200 μl H$_2$O, the phases were separated and the aqueous

35   phase extracted once with chloroform. The RNA was
precipitated with 1/5 volume 3 M NaOAc, 20 μg carrier
tRNA, and 2.5 volumes ethanol. The pellet was washed

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once with 70% ethanol, dried, and resuspended in 5 μl
H₂O and 5 μl formamide loading dye. The remainder of
the alkaline hydrolysis reaction was diluted 1:10 and
an equal volume of loading dye was added. To locate
5   where on the sequence ladder the boundary existed, an
RNase T1 digest of the ligand was electrophoresed
alongside the alkaline hydrolysis reaction and binding
reactions. The digest was done in a 10 μl reaction
containing 500 fmoles end-labeled RNA and 10 units
10   RNase T1 in 7 M urea, 20 mM Na-citrate (pH 5.0) and 1
mM EDTA. The RNA was incubated 10 minutes at 50°C
without enzyme and then another 10 minutes after adding
enzyme. The reaction was slowed by adding 10 μl
loading dyes and incubating at 4°C. Immediately after
15   digestion, 5 μl of each of the digest, hydrolysis, and
3 binding reactions were electrophoresed on a 12%
sequencing gel.

       <u>In vitro transcription of RNA 2-NH₂ ribose
derivatives of UTP and CTP</u>. RNA was transcribed
20   directly from the pUC18 plasmid miniprep dsDNA template
with T7 RNA polymerase in a reaction containing ATP,
GTP, 2NH₂-UTP and 2NH₂-CTP. For ³²P-labeled RNA, ³²P-ATP
was included in the reaction. Unmodified RNAs were
transcribed in a mixture containing ATP, GTP, UTP, and
25   CTP.

       <u>Synthesis of RNA</u>. RNA molecules corresponding to
lower limits of nucleotide sequence required for high
affinity binding to thrombin as determined by the
boundary experiments (Figure 30B) were synthesized on
30   an Applied Biosystems 394 DNA/RNA Synthesizer. These
RNA molecules include the class I clone 16 hairpin
structures of 24 nucleotides (24R) and 39 nucleotides
(39R) and the class II clone 27 hairpin of 33
nucleotides (33R).

35   <u>Binding of individual RNA molecules</u>. Four DNA
plasmids with unique 30N sequences were chosen for <u>in
vitro</u> transcription. ³²P-labelled RNA was transcribed

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with conventional nucleotides as well as with the 2-NH_2
derivatives of CTP and UTP. Binding curves with these
individual RNAs could be established using the binding
buffer and thrombin (1000 units, Sigma) concentrations

5   from 1.0×10^{-5} to 1.0×10^{-10} M. Human α thrombin
(Enzyme Research Laboratories, ERL) was also used to
determine binding affinities of RNA at concentrations
from 1.0×10^{-6} to 1.0×10^{-10} M.

Binding of the 5' end-labeled single stranded

10   15mer DNA 5'-GGTTGGTGTGGTTGG-3' (G15D) (SEQ ID NO:1)
described by Bock et al. (1992) supra, was determined
under the binding conditions described herein with ERL
thrombin and compared to binding by the radiolabelled
RNA hairpin structures described above.

15   Competition Experiments. To determine whether the
RNA ligands described can compete for binding of the
DNA 15mer G15D to thrombin, equimolar concentrations (1
μM) of thrombin and the 5' end labeled DNA 15mer G15D
were incubated under filter binding conditions (kD of

20   approximately 200 nM) in the presence and absence of
'cold' unlabeled RNA or DNA ligand at varying
concentrations from 10 nM to 1 uM. In the absence of
competition, RNA binding was 30%. The protein was
added last so competition for binding could occur. The

25   RNA ligands tested for competition were the class I
clone 16 synthetic RNAs 24mer (24R) and 39mer hairpins
(39R) and the class II 27 synthetic RNA 33mer (33R).
Results are expressed as the relative fraction of G15D
bound (G15 with competitor/G15 without competitor) vs.

30   the concentration of cold competitor.

To determine whether class I RNAs can compete for
binding with class II RNAs and to confirm the
competition with the G15D DNA, equimolar concentrations
(300 nM) of thrombin and the 5' end-labelled class II

35   RNA 33 hairpin were incubated under filter binding
conditions in the presence or absence of 'cold'
unlabelled RNA 24 or DNA G15D at varying concentrations

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from 100 nM to 32 μM. Results are expressed as the
relative fraction of RNA 33 bound (RNA 33 with
competitor/RNA 33 without competitor) versus the
concentration of cold competitor (Figure 33).

Chromogenic assay for thrombin activity and
inhibition by RNA ligands. The hydrolysis by thrombin
of the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-
pNitroaniline [H-D-Phe-Pip-Arg-pNA]) (Kabi Pharmacia)
was measured photometrically at 405 nm due to the
release of p-nitroaniline (pNA) from the substrate.

$$\text{H-D-Phe-Pip-Arg-pNA} + H_2O \xrightarrow{\text{Thrombin}} \text{H-D-Phe-Pip-Arg-OH} + \text{pNA}$$

Thrombin was added to a final concentration of 10^{-8} or
10^{-9} M to a reaction buffer (50 mM Na citrate, pH 6.5,
150 mM NaCl, 0.1% PEG), containing 250 μM S2238
substrate at 37°C. For inhibition assays, thrombin
plus RNA (equimolar or at 10-fold excess) were
preincubated 30 secs at 37°C before adding to the
reaction mixture (Figure 34A).

Fibrinogen clotting. Thrombin was added for a
final concentration of 2.5 nM to 400 μl incubation
buffer (20 mM Tris-acetate, pH 7.4, 140 mM NaCl, 5 mM
KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) containing 0.25 mg/ml
fibrinogen and 1 u/λ RNAse inhibitor (RNAasin, Promega)
with or without 30 nM RNA class I or 60 nM RNA class II
at 37°C. Time in seconds from addition of thrombin to
clot formation was measured by the tilt test (Figure
34B).

Specificity of Thrombin Binding. The binding
affinity of the full-length class I RNA 16, class II
RNA 27 and bulk 30N3 RNA for the serum proteins
Antithrombin III (ATIII) and Prothrombin was determined
by filter binding, as described above for the evolution
of high affinity RNA ligands. These experiments were
done in protein excess at concentrations from 1 x 10^{-5}
to 5 x 10^{-10} M at a final RNA concentration of 2 x 10^{-9} M

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(Figure 35).

EXAMPLE V. NUCLEIC ACID LIGANDS TO bFGF.

Materials. bFGF was obtained from Bachem
California (molecular weight 18,000 Da, 154 amino
acids). Tissue culture grade heparin (average
molecular weight 16,000 Da) was purchased form Sigma.
Low molecular weight heparin (5,000 Da) was from
Calbiochem. All other chemicals were at least reagent
grade and were purchased from commercial sources.

SELEX. Essential features of the SELEX protocol
have been described in detail in previous papers (Tuerk
& Gold (1990) supra; Tuerk et al. (1992a) supra; Tuerk
et al. (1992b) in Polymerase Chain Reaction (Ferre, F,
Mullis, K., Gibbs, R. & Ross, A., eds.) Birkhauser,
NY). Briefly, DNA templates for in vitro transcription
(that contain a region of thirty random positions
flanked by constant sequence regions) and the
corresponding PCR primers were synthesized chemically
(Operon). The random region was generated by utilizing
an equimolar mixture of the four nucleotides during
oligonucleotide synthesis. The two constant regions
were designed to contain PCR primer annealing sites, a
primer annealing site for cDNA synthesis, T7 RNA
polymerase promoter region, and restriction enzyme
sites that allow cloning into vectors (See Table I).

An initial pool of RNA molecules was prepared by
in vitro transcription of about 200 picomoles (pmol)
(10^{14} molecules) of the double stranded DNA template
utilizing T7 RNA polymerase (New England Biolabs).
Transcription mixtures consisted of 100-300 nM
template, 5 units/ul T7 RNA polymerase, 40 mM Tris-Cl
buffer (pH 8.0) containing 12 mM $MgCl_2$, 5 mM DTT, 1 mM
spermidine, 0.002% Triton X-100, and 4% PEG.
Transcription mixtures were incubated at 37°C for 2-3
hours. These conditions typically resulted in
transcriptional amplification of 10- to 100-fold.

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Selections for high affinity RNA ligands were done
by incubating bFGF (10-100 pmol) with RNA (90-300 pmol)
for 10 minutes at 37°C in 50 ul of phosphate buffered
saline (PBS)(10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM
NaCl, 2.7 mM KCl, pH 7.4), then separating the protein-
RNA complexes from the unbound species by
nitrocellulose filter partitioning (Tuerk & Gold (1990)
supra). The selected RNA (which typically amount to
0.3-8% of the total input RNA) was then extracted from
the filters and reverse transcribed into cDNA by avian
myeloblastosis virus reverse transcriptase (AMV RT,
Life Sciences). Reverse transcriptions were done at
48°C (30 minutes) in 50 mM Tris buffer (pH 8.3), 60 mM
NaCl, 6 mM $Mg(OAc)_2$, 10 mM DTT, and 1 unit/ul AMV RT.
Amplification of the cDNA by PCR under standard
conditions yielded sufficient amounts of double-
stranded DNA for the next round of in vitro
transcription.

Nitrocellulose Filter Binding Assay.
Oligonucleotides bound to proteins can be effectively
separated from the unbound species by filtration
through nitrocellulose membrane filters (Yarus & Berg
(1970) Anal. Biochem. 35:450; Lowary & Uhlenbeck (1987)
Nucleic Acids Res. 15:10483; Tuerk & Gold (1990)
supra). Nitrocellulose filters (Millipore, 0.45 um
pore size, type HA) were secured on a filter manifold
and washed with 4-10 ml of buffer. Following
incubations of ^{32}P-labeled RNA with serial dilutions of
the protein (5-10 min) at 37°C in buffer (PBS)
containing 0.01% human serum albumin (HSA), the
solutions were applied to the filters under gentle
vacuum in 45 ul aliquots and washed with 5 ml of PBS.
The filters were then dried under an infrared lamp and
counted in a scintillation counter.

Cloning and Sequencing. Individual members of the
enriched pools were cloned into pUC18 vector and
sequenced as described (Schneider et al. (1992) supra;

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Tuerk & Gold (1990) supra.

SELEX Experiments Targeting bFGF. Following the
procedures described above, two SELEX experiments
(Experiments A and B) targeting bFGF were initiated
with separate pools of randomized RNA, each pool
consisting of approximately 10^{14} molecules. The
constant sequence regions that flank the randomized
region, along with the corresponding primers, were
different in each experiment. The two template/primer
combinations used are shown in Table I.

Selections were conducted in PBS at 37°C. The
selection conducted in Experiment B was done in the
presence of heparin (Sigma, molecular weight 5,000-
32,000 Da, average molecular weight 16,000 Da) in the
selection buffer at the molar ration of 1/100
(heparin/bFGF). Heparin competes for binding of
randomized RNA to bFGF and the amount of heparin. The
amount of heparin used significantly reduced but did
not eliminate RNA binding to bFGF (data not shown).
The rationale for using heparin was two-fold. First,
heparin is known to induce a small conformational
change in the protein and also stabilizes bFGF against
thermal denaturation. Second, the apparent competitive
nature of binding of heparin with randomized RNA to
bFGF was expected to either increase the stringency of
selection for the heparin binding site or direct the
binding of RNA ligands to alternative site(s).

Significant improvement in affinity of RNA ligands
to bFGF was observed in Experiment A after ten rounds,
and in Experiment B after thirteen rounds. Sequencing
of these enriched pools of RNA ligands revealed a
definite departure from randomness which indicated that
the number of molecules remaining in the pool was
substantially reduced. Individual members of the
enriched pools were then cloned into pUC18 vector and
sequenced as described above.

49 clones were sequenced from Experiment A, and 37

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clones from Experiment B. From the total of 86
sequences, 71 were unique. Two distinct families could
be identified based on overlapping regions of sequence
homology (Tables II and III). A number of sequences
with no obvious homology to members of either of the
two families were also present, as expected (Irvine et
al.(1991) J. Mol. Biol. 222:739), and are shown in
Table IV.

The consensus sequence from family 1 ligands
(Table II) is defined by a contiguous stretch of 9
bases, CUAACCAGG (SEQ ID NO:27). This suggests a
minimal structure consisting of a 4-5 nucleotide loop
that includes the strongly conserved AACC sequence and
a bulged stem (Figure 41 and Table VI). The consensus
sequence for family 2 ligands (Table III) is more
extended and contains less conserved regions,
RRGGHAACGYWNNGDCAAGNNCACYY (SEQ ID NO:43). Here, most
of the strongly conserved positions are accommodated in
a larger (19-21 nucleotide) loop (Figure 41 and Table
VII). Additional structure within the loop is
possible.

The existence of two distinct sequence families in
the enriched pools of RNA suggest that there are two
convergent solutions for high-affinity binding to bFGF.
SELEX experiment A contributed members to both sequence
families (Table II). All of the sequences from the
SELEX experiment B (selected in the presence of
heparin), on the other hand, belong either to family 2
(Table III) or to the "other sequences" family (Table
IV) family, but none were found in family 1. This is
surprising in view of the fact that bFGF was present in
a formal molar excess of 100-fold over heparin during
selections. The effective molar excess of bFGF over
heparin, however, was probably much smaller. Average
molecular weight of heparin used in selections was
16,000 Da. Since each sugar unit weighs 320 Da and at
least eight sugar units are required for high-affinity

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binding to bFGF, six molecules of bFGF, on average, can
bind to a molecule of heparin. This reduces the molar
ratio of heparin to bFGF to 1:16. In practice, this
amount of heparin is sufficient to reduce the observed
affinity of the unselected RNA pool for bFGF by a
factor of five (data not shown). The observed
exclusion of an entire ligand family by presence of a
relatively small amount of heparin in the selection
buffer may be a consequence of a conformational change
in the protein induced by heparin. Because of the
relative amounts of heparin and bFGF that were used in
selections, this model requires that the heparin-
induced conformation persist after the protein-heparin
complex has dissociated, and that the lifetime of this
conformer is long enough to permit equilibration with
the RNA ligands.

Family 2 sequences are comprised of clones derived
from both SELEX experiments. This suggests that the
flanking constant regions typically play a relatively
minor role in determining the affinity of these ligands
and supports the premise that the consensus sequence in
this family is the principal determinant of high-
affinity binding to bFGF.

Determination of Binding Affinities for bFGF.

Equilibrium Dissociation Constants. In the
simplest case, equilibrium binding of RNA to bFGF can
be described by equation 1:

$$RNA \bullet bFGF \rightleftharpoons RNA + bFGF \qquad (1)$$

The fraction of bound RNA (q) is related to the
concentration of free protein, [P] (equation 2):

$$q = f[P]/([P] + K_d) \qquad (2)$$

where K_d is the equilibrium dissociation constant and f
reflects the efficiency of retention of the protein-RNA

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complexes on nitrocellulose filters. Mean value of f
for bFGF was 0.82.

In order to eliminate higher order structures, all
RNA solutions were heated to 90°C in PBS for 2-3
minutes and cooled on ice prior to incubation with
protein. Only single bands for all RNA clones were
detected on non-denaturing polyacrylamide gels
following this treatment.

Relative binding affinity of individual ligands to
bFGF cannot be predicted from sequence information.
Unique sequence clones were therefore screened for
their ability to bind to bFGF by measuring the fraction
of radiolabeled RNA bound to nitrocellulose filters
following incubation with 4 and 40 nM protein. This
screening method was sufficiently accurate to allow
several clones to be identified that had dissociation
constants in the nanomolar range. Binding of these
select clones was then analyzed in more detail.

High-affinity RNA ligands for bFGF were found in
both sequence families (Tables VI and VII). The
affinity of clones that did not belong to either family
was generally lower (data not shown).

The original, unselected RNA pools bound to bFGF
with 300 nM (set A) and 560 nM (set B) affinities
(Figure 36). SELEX therefore allowed the isolation of
ligands with at least 2 orders of magnitude better
affinity for bFGF.

In order to address the question of specificity, a
representative set of high-affinity ligands for bFGF
(5A and 7A from family 1; 12A and 26A from family 2)
was tested for binding to four other heparin-binding
proteins. It was found that the affinity of these
ligands for acidic FGF, thrombin, antithrombin III, and
vascular endothelial growth factor was relatively weak
(K_d > 0.3 uM)(data not shown).

RNA Ligand Inhibition of bFGF Receptor Binding.
The same four high-affinity RNA ligands were also

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tested for their ability to inhibit binding of bFGF to
the low- and the high-affinity cell-surface receptors.

Receptor Binding Studies. bFGF was labeled with
^{125}I by the Iodo-Gen (Pierce) procedure as described by
Moscatelli (1987) supra. Confluent baby hamster kidney
(BHK) cells were washed extensively with PBS and then
incubated for 2 hours at 4°C with αMEM medium
containing 10 ng/ml ^{125}I-bFGF in PBS, 0.1% HSA, 1
unit/ml RNasein, and serial dilutions of high-affinity
RNA. In a separate experiment it was established that
the RNA is not significantly degraded under these
conditions. The amount of ^{125}I-bFGF bound to the low-
and the high-affinity receptor sites was determined as
described by Moscatelli (1987) supra.

All four ligands competed for the low-affinity
receptor sites while the unselected (random) RNAs did
not (Figure 37A). The concentration of RNA required to
effect half-displacement of bFGF from the low-affinity
receptor was 5-20 nM for ligands 5A, 7A and 26A, and
>100 nM for ligand 12A. Half-displacement from the
high-affinity sites is observed at the concentration of
RNA near 1 uM for ligands 5A, 7A and 26A, and > 1 uM
for ligand 12A. Again, random RNAs did not compete for
the high-affinity receptor. The observed difference in
concentration of RNA required to displace bFGF from the
low- and high-affinity receptors is expected as a
reflection of the difference in affinity of the two
receptor classes for bFGF (2-10 nM for the low-affinity
sites and 10-100 pM for the high-affinity sites).

Heparin competitively displaced RNA ligands from
both sequence families (Figure 38), although higher
concentrations of heparin were required to displace
members of family 2 from bFGF.

The selective advantage obtained through the SELEX
procedure is based on affinity to bFGF. RNA ligands
can in principle bind to any site on the protein, and
it is therefore important to examine the activity of

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the ligands in an appropriate functional assay. The
relevant functional experiment for the selected high-
affinity ligands is testing their ability to inhibit
binding of bFGF to its cell-surface receptors since
this is how bFGF exerts its biological activity. The
fact that several representative high-affinity RNA
ligands inhibited binding of bFGF to both receptor
classes (in accord with their relative binding
affinities) suggests that these ligands bind at or near
the receptor binding site(s). Further support for this
notion comes from the observation that heparin competes
for binding of these ligands to bFGF. High affinity
ligands from family 1 and family 2 may bind to
different sites on bFGF. This invention includes
covalently connecting components from the two ligand
families into a single, more potent inhibitor of bFGF.

TABLE I. OLIGONUCLEOTIDES USED IN SELEX EXPERIMENTS A AND B.

EXPERIMENT A	SEQUENCE 5'-3'	SEQ ID NUMBER
Starting RNA	GGGAGCUCAGAAUAAACGCUCAANNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNUUCGACA UGAGGCCCGGAUCCGGC	SEQ ID NO:21
PCR Primer 1	HindIII CCGAAGCTTAATACGACTCACTATAGGGAG T7 Promoter CTCAGAATAAACGCTCAA	SEQ ID NO:22
PCR Primer 2	BamH1 GCCGGATCCGGGCCTCATGTCGAA	SEQ ID NO:23
EXPERIMENT B		
Starting RNA	GGGAGAUGCCUGUCGAGCAUGCUGNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNGUAGCUAA ACAGCUUUGUCGACGGG	SEQ ID NO:24
PCR Primer 1	HindIII CCCGAAGCTTAATACGACTCACTATAGGGAG T7 Promoter ATGCCTGTCGAGCATGCTG	SEQ ID NO:25
PCR Primer 2	Sal1 CCCGTCGACAAAGCTGTTTAGCTAC	SEQ ID NO:26

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TABLE II. FAMILY 1 SEQUENCES OF THE RANDOM REGION FROM SELEX
          EXPERIMENT A AND B.

FAMILY 1	CONSENSUS SEQUENCE CUAACCNGG (SEQ ID NO:27)	SEQ ID NUMBER
4A	UGCUAUUCGCCUAACUCGGCGCUCCUACCU	SEQ ID NO:28
5A	AUCUCCUCCCGUCGAAGCUAACCUGGCCAC	SEQ ID NO:29
7A	UCGGCGAGCUAACCAAGACACUCGCUGCAC	SEQ ID NO:30
10A	GUAGCACUAUCGGCCUAACCCGGUAGCUCC	SEQ ID NO:31
13A	ACCCGCGGCCUCCGAAGCUAACCAGGACAC	SEQ ID NO:32
14A	UGGGUGCUAACCAGGACACACCCACGCUGU	SEQ ID NO:33
16A	CACGCACAGCUAACCAAGCCACUGUGCCCC	SEQ ID NO:34
18A	CUGCGUGGUAUAACCACAUGCCCUGGGCGA	SEQ ID NO:35
21A	UGGGUGCUUAACCAGGCCACACCCUGCUGU	SEQ ID NO:36
25A	CUAGGUGCUAUCCAGGACUCUCCCUGGUCC	SEQ ID NO:37
29A	UGCUAUUCGCCUAGCUCGGCGCUCCUACCU	SEQ ID NO:38
38A	AGCUAUUCGCCCAACCCGGCGCUCCCGACC	SEQ ID NO:39
39A	ACCAGCUGCGUGCAACCGCACAUGCCUGG	SEQ ID NO:40
56A	CAGGCCCCGUCGUAAGCUAACCUGGACCCU	SEQ ID NO:41
61A	UGGGUGCUAACCACCACACACUCACGCUGU	SEQ ID NO:42

TABLE III.   FAMILY 2 SEQUENCES OF THE RANDOM REGION FROM SELEX
              EXPERIMENTS A AND B.

FAMILY 2	CONSENSUS SEQUENCE: RRGGHAACGYWNNGDCAAGNNCACYY (SEQ ID NO:43)	SEQ ID NUMBER
11A	GGGUAACGUUGU GACAAGUACACCUGCGUC	SEQ ID NO:44
12A	GGGGCAACGCUACA GACAAGUGCACCCAAC	SEQ ID NO:45
26A	CGUCAGAAGGCAACGUAUA GGCAAGCACAC	SEQ ID NO:46
27A	CCUCUCGAAGACAACGCUGU GACAAG ACAC	SEQ ID NO:47
47A	AGUGGGAAACGCUACUUGACAAG ACACCAC	SEQ ID NO:48
65A	GGCUACGCUAAU GACAAGUGCACUUGGGUG	SEQ ID NO:49
1B	CUCUGGUAACGCAAU GUCAAGUGCACAUGA	SEQ ID NO:50
2B	AGCCGCAGGUAACGGACC GGCGAGACCAUU	SEQ ID NO:51
6B	ACGAGCUUCGUAACGCUAUC GACAAGUGCA	SEQ ID NO:52
8B	AAGGGGAAACGUUGA GUCCGGUACACCCUG	SEQ ID NO:53
9B	AGGGUAACGUACU GGCAAGCUCACCUCAGC	SEQ ID NO:54
11B	GAGGUAACGUAC GACAAGACCACUCCAACU	SEQ ID NO:55
12B	AGGUAACGCUGA GUCAAGUGCACUCGACAU	SEQ ID NO:56
13B	GGGAAACGCUAUC GACGAGUGCACCCGGCA	SEQ ID NO:57
14B	CCGAGGGUAACGUUGG GUCAAGCACACCUC	SEQ ID NO:58
15B	UCGGGGUAACGUAUU GGCAAGGC ACCCGAC	SEQ ID NO:59
19B	GGUAACGCUGUG GACAAGUGCACCAGCUGC	SEQ ID NO:60
22B	AGGGUAACGUACU GGCAAGCUCACCUCAGC	SEQ ID NO:61
28B	AGGGUAACGUAUA GUCAAGAC ACCUCAAGU	SEQ ID NO:62
29B	GGGUAACGCAUU GGCAAGAC ACCCAGCCCC	SEQ ID NO:63
36B	GAGGAAACGUACC GUCGAGCC ACUCCAUGC	SEQ ID NO:64
38B	AGGUAACGCUGA GUCAAGUGCACUCGACAU	SEQ ID NO:65
48B	GGGUAACGUGU GACAAGAUCACCCAGUUUG	SEQ ID NO:66
49B	CACAGGGCAACGCUGCU GACAAGUGCACCU	SEQ ID NO:67

TABLE IV. OTHER SEQUENCES OF THE RANDOM REGION FROM SELEX
          EXPERIMENTS A AND B.

NUMBER	SEQUENCE	SEQ ID NUMBER
8A	ACGCCAAGUGAGUCAGCAACAGAGCGUCCG	SEQ ID NO:68
9A	CCAGUGAGUCCUGGUAAUCCGCAUCGGGCU	SEQ ID NO:69
24A	CUUCAGAACGGCAUAGUGGUCGGCCGCGCC	SEQ ID NO:70
33A	AGGUCACUGCGUCACCGUACAUGCCUGGCC	SEQ ID NO:71
34A	UCCAACGAACGGCCCUCGUAUUCAGCCACC	SEQ ID NO:72
36A	ACUGGAACCUGACGUAGUACAGCGACCCUC	SEQ ID NO:73
37A	UCUCGCUGCGCCUACACGGCAUGCCGGGA	SEQ ID NO:74
40A	GAUCACUGCGCAAUGCCUGCAUACCUGGUC	SEQ ID NO:75
43A	UCUCGCUGCGCCUACACGGCAUGCCCGGGA	SEQ ID NO:76
44A	UGACCAGCUGCAUCCGACGAUAUACCCUGG	SEQ ID NO:77
45A	GGCACACUCCAACGAGGUAACGUUACGGCG	SEQ ID NO:78
55A	AGCGGAACGCCACGUAGUACGCCGACCCUC	SEQ ID NO:79
4B	ACCCACGCCCGACAACCGAUGAGUUCUCGG	SEQ ID NO:80
5B	UGCUUUGAAGUCCUCCCCGCCUCUCGAGGU	SEQ ID NO:81
7B	AUGCUGAGGAUAUUGUGACCACUUCGGCGU	SEQ ID NO:82
16B	ACCCACGCCCGACAACCGAUGAGCUCGGA	SEQ ID NO:83
20B	AGUCCGGAUGCCCCACUGGGACUACAUUGU	SEQ ID NO:84
21B	AAGUCCGAAUGCCACUGGGACUACCACUGA	SEQ ID NO:85
23B	ACUCUCACUGCGAUUCGAAAUCAUGCCUGG	SEQ ID NO:86
40B	AGGCUGGGUCACCGACAACUGCCCGCCAGC	SEQ ID NO:87
42B	AGCCGCAGGUAACGGACCGGCGAGACCACU	SEQ ID NO:88
26B	GCAUGAAGCGGAACUGUAGUACGCGAUCCA	SEQ ID NO:89

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TABLE V.   REPEAT SEQUENCES OF THE RANDOM REGION FROM SELEX
           EXPERIMENTS A AND B.

NUMBER	SEQUENCE	SEQUENCE ID NUMBER	CLONE REPEATED
3A	GGGUAACGUUGUGACAAGUACACCUGCGUU	SEQ ID NO:90	11A
15A	GGGUAACGUUGUGACAAGUACACCUGCGUC	SEQ ID NO:91	11A
20A	GGGUAACGUUGUGACAAGUACACCUGCGUC	SEQ ID NO:92	11A
48A	GGGUAACGUUGUGACAACUACACCUGCGUC	SEQ ID NO:93	11A
58A	GGGUAACGUUGUGACAACUACACCUGCGUC	SEQ ID NO:94	11A
64A	GGGUAACGUUGUGACAACUACACCUGCGUC	SEQ ID NO:95	11A
28A	CGUCAGAAGGCAACGUAUAGGCAAGCACAC	SEQ ID NO:96	26A
30A	GUAGCACUAUCGGCCUAACCCGGUAGCUCC	SEQ ID NO:97	10A
23A	ACCCGCGGCCUCCGAAGCUAACCAGGACAC	SEQ ID NO:98	13A
46A	AGGUCACUGCGUCACCGUACAUGCCUGGCC	SEQ ID NO:99	33A
49A	AGGUCACUGCGUCACCGUACAUGCCUGGCC	SEQ ID NO:100	33A
50A	GGCACACUCCAACGAGGUAACGUUACGGCG	SEQ ID NO:101	45A
41A	GGGGCAACGCUACAGACAAGUGCACCCAAC	SEQ ID NO:102	12A
51A	GGGGCAACGCUACAGACAAGUGCACCCAAC	SEQ ID NO:103	12A
54A	GGGGCAACGCUACAGACAAGUGCACCCAAC	SEQ ID NO:104	12A
35A	UGGGUGCUAACCAGGACACACCCACGCUGU	SEQ ID NO:105	14A
18B	CCGAGGGUAACGUUGGGUCAAGCACACCUC	SEQ ID NO:106	14B
24B	GGGAAACGCUAUCGACGAGUGCACCCGGCA	SEQ ID NO:107	13B
39B	GGGAAACGCUAUCGACGAGUGCACCCGGCA	SEQ ID NO:108	13B
37B	ACUCUCACUGCGAUUCGAAAUCAUGCCUGG	SEQ ID NO:109	23B
43B	GCAUGAAGCGGAACUGUAGUACGCGAUCCA	SEQ ID NO:110	26B
46B	GCAUGAAGCGGAACUGUAGUACGCGAUCCA	SEQ ID NO:111	26B
25B	AGGGUAACGUACUGGCAAGCUCACCUCAGC	SEQ ID NO:112	9B
33B	AGGGUAACGUACUGGCAAGCUCACCUCAGC	SEQ ID NO:113	9B
31B	GGUAACGCUGUGGACAAGUGCACCAGCUGC	SEQ ID NO:114	19B

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TABLE VI. SECONDARY STRUCTURES AND DISSOCIATION CONSTANTS (K_d's) FOR A REPRESENTATIVE SET OF HIGH-AFFINITY LIGANDS FROM FAMILY 1.

LIGAND	STRUCTURE[a]	Kd, nM
| 5A | ```
        CC              AA
   CCUC   GUCGAA---GCU   C
   ggag   cagcuu    CGG  C
     ua          CAC   U
``` | 23 ± 3 |
| 7A | ```
                   AA
         CGGCGAG---CU  C
         GUCGCUC   GA  C
                  ACA  A
``` | 5.0 ± 0.5 |
| 13A | ```
  C                        A
CCG GGCCUC----CGAAG----CU  A
ggc-ccggag    gcuuC    GA  C
        uaca       ACAG  C
``` | 3.2 ± 0.5 |
| 14A | ```
    cucaa              A
 aaacg    UGGGUG----CU A
 uuUGU-   -ACCCAC   GA C
     CGC        ACAG  C
``` | 3.0 ± 0.5 |
| 21A | ```
                    A
  aaU----GGGU---GCUU A
  uUG    CCCA   CGGA C
   UCGU       CAC   C
``` | 8.1 ± 0.8 |
| 25A | ```
                 A
   CUA-GGUG----CU U
   GGU CCUC    GA C
     C     UCAG  C
``` | 5.9 ± 1.4 |
| 39A | ```
       CU         A
  AACCAG  GC--GUGC A
  uuGGUC--CG  CACG C
         UA      C
``` | 8.5 ± 1.2 |

[a]Strongly conserved positions are shown in boldface symbols. Nucleotides in the constant region are in lowercase type.

TABLE VII. SECONDARY STRUCTURES AND DISSOCIATION CONSTANTS (K$_d$'s) FOR A REPRESENTATIVE SET OF HIGH-AFFINITY LIGANDS FROM FAMILY 2.

LIGAND	STRUCTURE[a]	Kd, nM

12A	<pre> CAACGCU
	        G A
	                  C
	uc-aa---GGG A
	ag uu CCC G
	  c CAA A A
	        CGUGAAC</pre>
		0.9 ± 0.2

26A	<pre> CAACGUA
	      A G U
	     GUC GAAG A
	     cag-cuuC G
	          A G
	        CACGAAC</pre>
		0.4 ± 0.1

65A	<pre> CUACGUA
	        G A
	                  A
	aacgcucaaG U
	uuGUGGGUUC G
	        A A
	        CGUGAAC</pre>
		0.6 ± 0.04

22B	<pre> UAACGUA
	          G C
	agc-augcugAGG U
	ucg ugCGACUCC G
	   a A G
	          CUCGAAC</pre>
		1 ± 0.6

28B	<pre> UAACGUA
	         G U
	augc-ugAGG
	ugUG ACUCC A
	    A A G
	        CAGAACU</pre>
		2 ± 1

38B	<pre> UAACGCU
	       c G G
	gcaug ugAG . A
	ugUAC GCUC G
	    A A U
	        CGUGAAC</pre>
		4 ± 1

2B	<pre> UAACGCA
	      C G C
	    AGC GCAG C
	    ucg ugUU G
	     a A G
	        CCAGAGC</pre>
		170 ± 80

[a]Strongly conserved positions are shown in boldface symbols. Nucleotides in the constant region are in lowercase type.

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SEQUENCE LISTING
(1) GENERAL INFORMATION:
   (i) APPLICANT: Larry Gold
                        Craig Tuerk
                        Diane Tasset
                        Nebojsa Janjic

   (ii) TITLE OF INVENTION: Nucleic Acid Ligands and Methods for
                            Producing the Same

   (iii) NUMBER OF SEQUENCES: 159
   (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Beaton & Swanson, P.C.
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        (C) CITY: Denver
        (D) STATE: Colorado
        (E) COUNTRY: USA
        (F) ZIP: 80237

   (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Diskette, 3.50 inch, 800 Kb storage
        (B) COMPUTER: IBM
        (C) OPERATING SYSTEM: MS-DOS
        (D) SOFTWARE: WordPerfect 5.1

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

   (vii)PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/714,131
        (B) FILING DATE: 10-JUNE-1991

   (vii)PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/536,428
        (B) FILING DATE: 11-JUNE-1990

   (vii)PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 08/061,691
        (B) FILING DATE: 22-APRIL-1993

   (vii)PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/973,333
        (B) FILING DATE: 06-NOVEMBER-1992

   (vii)PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/964,624
        (B) FILING DATE: 21-OCTOBER-1992

   (vii)PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/953,694
        (B) FILING DATE: 29-SEPTEMBER-1992

   (viii)ATTORNEY/AGENT INFORMATION:
        (A) NAME: Barry J. Swanson
        (B) REGISTRATION NUMBER: 33,215
        (C) REFERENCE/DOCKET NUMBER: NEX03/PCT

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```
    (ix)  TELECOMMUNICATION INFORMATION:
          (A)   TELEPHONE:  (303) 850-9900
          (B)   TELEFAX:  (303) 850-9401

(2)  INFORMATION FOR SEQ ID NO:1:

     (i)  SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 15 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:1:
```

GGTTGGTGTG GTTGG   15

```
(3)  INFORMATION FOR SEQ ID NO:2:
     (i)  SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 31 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:2:
```

AAAAAUCCGA AGUGCAACGG GAAAAUGCAC U   31

```
(4)  INFORMATION FOR SEQ ID NO:3:
     (i)  SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 85 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:3:
```

CCCGGATCCT CTTTACCTCT GTGTGAGATA CAGAGTCCAC AAACGTGTTC TCAATGCACC 60
CGGTCGGAAG GCCATCAATA GTCCC   85

```
(5)  INFORMATION FOR SEQ ID NO:4:
     (i)  SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 49 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:4:
```

CCGAAGCTTA ATACGACTCA CTATAGGGAC TATTGATGGC CTTCCGACC   49

```
(6)  INFORMATION FOR SEQ ID NO:5:
     (i)  SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 25 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:5:
```

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CCCGGATCCT CTTTACCTCT GTGTG   25

(7) INFORMATION FOR SEQ ID NO:6:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

UUCCGNNNNN NNNCGGGAAA A   21

(8) INFORMATION FOR SEQ ID NO:7:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 24 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

UCCGNNNNNN NNCGGGAAAA NNNN   24

(9) INFORMATION FOR SEQ ID NO:8:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 27 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

NNUUCCGNNN NNNNNCGGGA AAANNNN   27

(10) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGAUCGGAAN NAGUAGGC   18

(11) INFORMATION FOR SEQ ID NO:10:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCGGCUUUGG GCGCCGUGCU U   21

(12) INFORMATION FOR SEQ ID NO:11:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 57 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGUCCGAAGU GCAACGGGAA AAUGCACUAU GAAAGAAUUU UAUAUCUCUA UUGAAAC   57

(13) INFORMATION FOR SEQ ID NO:12:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 31 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGGATCCGT TTCAATAGAG ATATAAAATT C   31

(14) INFORMATION FOR SEQ ID NO:13:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 55 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGCAAGCTT TAATACGACT CACTATAGGT CCGAAGTGCA ACGGGAAAAT GCACT   55

(15) INFORMATION FOR SEQ ID NO:14:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 31 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTTCAATAG AGATATAAAA TTCTTTCATA G   31

(16) INFORMATION FOR SEQ ID NO:15:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 89 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTTCAATAG AGATATAAAA TTCTTTCATA GNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 60
NAGTGCATTT TCCCGTTGCA CTTCGGACC   89

(17) INFORMATION FOR SEQ ID NO:16:
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 83 base pairs

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```
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCCGGATCCT CTTTACCTCT GTGTGAGATA CAGAGTCCAC AACGTGTTCT CAATGACCCG 60
GTCGGAAGGC CATCAATAGT CCC   83

```
(18) INFORMATION FOR SEQ ID NO:17:
     (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 50 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCGAAGCTTA ATACGACTCA CTATAGGGAC TATTGATGGG CCTTCCGACC   50

```
(19) INFORMATION FOR SEQ ID NO:18:
     (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 48 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear
```

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCGAAGCTTA ATACGACTCA CTATAGGGAG CTCAGAATAA ACGCTCAA   48

```
(20) INFORMATION FOR SEQ ID NO:19:
     (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 87 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCCGGATCCG GGCCTCATGT CGAANNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 60
NNNNTTGAGC GTTTATTCTG AGCTCCC   87

```
(21) INFORMATION FOR SEQ ID NO:20:
     (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 24 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCCGGATCCG GGCCTCATGT CGAA   24

```
(22) INFORMATION FOR SEQ ID NO:21:

     (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 77 base pairs
```

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```
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear
```

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGGAGCUCAG AAUAAACGCU CAANNNNNNN NNNNNNNNNN NNNNNNNNNN NNNUUCGACA 60
UGAGGCCCGG AUCCGGC   77

(23) INFORMATION FOR SEQ ID NO:22:

```
       (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 48 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear
```

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCGAAGCTTA ATACGACTCA CTATAGGGAG CTCAGAATAA ACGCTCAA   48

(24) INFORMATION FOR SEQ ID NO:23:

```
       (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 24 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear
```

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCCGGATCCG GGCCTCATGT CGAA   24

(25) INFORMATION FOR SEQ ID NO:24:

```
       (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 79 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear
```

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGGAGAUGCC UGUCGAGCAU GCUGNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNGUAGCU 60
AAACAGCUUU GUCGACGGG   79

(26) INFORMATION FOR SEQ ID NO:25:

```
       (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 50 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear
```

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCCGAAGCTT AATACGACTC ACTATAGGGA GATGCCTGTC GAGCATGCTG   50

(27) INFORMATION FOR SEQ ID NO:26:

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    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 25 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCCGTCGACA AAGCTGTTTA GCTAC   25

(28) INFORMATION FOR SEQ ID NO:27:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 9 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CUAACCNGG   9

(29) INFORMATION FOR SEQ ID NO:28:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

UGCUAUUCGC CUAACUCGGC GCUCCUACCU   30

(30) INFORMATION FOR SEQ ID NO:29:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AUCUCCUCCC GUCGAAGCUA ACCUGGCCAC   30

(31) INFORMATION FOR SEQ ID NO:30:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

UCGGCGAGCU AACCAAGACA CUCGCUGCAC   30

(32) INFORMATION FOR SEQ ID NO:31:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GUAGCACUAU CGGCCUAACC CGGUAGCUCC   30

(33) INFORMATION FOR SEQ ID NO:32:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ACCCGCGGCC UCCGAAGCUA ACCAGGACAC   30

(34) INFORMATION FOR SEQ ID NO:33:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

UGGGUGCUAA CCAGGACACA CCCACGCUGU   30

(35) INFORMATION FOR SEQ ID NO:34:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CACGCACAGC UAACCAAGCC ACUGUGCCCC   30

(36) INFORMATION FOR SEQ ID NO:35:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CUGCGUGGUA UAACCACAUG CCCUGGGCGA   30

(37) INFORMATION FOR SEQ ID NO:36:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

UGGGUGCUUA ACCAGGCCAC ACCCUGCUGU   30

(38) INFORMATION FOR SEQ ID NO:37:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CUAGGUGCUA UCCAGGACUC UCCCUGGUCC   30

(39) INFORMATION FOR SEQ ID NO:38:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

UGCUAUUCGC CUAGCUCGGC GCUCCUACCU   30

(40) INFORMATION FOR SEQ ID NO:39:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGCUAUUCGC CCAACCCGGC GCUCCCGACC   30

(41) INFORMATION FOR SEQ ID NO:40:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 29 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ACCAGCUGCG UGCAACCGCA CAUGCCUGG   29

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(42) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CAGGCCCCGU CGUAAGCUAA CCUGGACCCU   30

(43) INFORMATION FOR SEQ ID NO:42:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

UGGGUGCUAA CCACCACACA CUCACGCUGU   30

(44) INFORMATION FOR SEQ ID NO:43:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

RRGGHAACGY WNNGDCAAGN NCACYY   26

(45) INFORMATION FOR SEQ ID NO:44:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGGUAACGUU GUGACAAGUA CACCUGCGUC   30

(46) INFORMATION FOR SEQ ID NO:45:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GGGGCAACGC UACAGACAAG UGCACCCAAC   30

(47) INFORMATION FOR SEQ ID NO:46

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CGUCAGAAGG CAACGUAUAG GCAAGCACAC   30

(48) INFORMATION FOR SEQ ID NO:47:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCUCUCGAAG ACAACGCUGU GACAAGACAC   30

(49) INFORMATION FOR SEQ ID NO:48:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

AGUGGGAAAC GCUACUUGAC AAGACACCAC   30

(50) INFORMATION FOR SEQ ID NO:49:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGCUACGCUA AUGACAAGUG CACUUGGGUG   30

(51) INFORMATION FOR SEQ ID NO:50:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CUCUGGUAAC GCAAUGUCAA GUGCACAUGA   30

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(52) INFORMATION FOR SEQ ID NO:51:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

AGCCGCAGGU AACGGACCGG CGAGACCAUU   30

(53) INFORMATION FOR SEQ ID NO:52:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

ACGAGCUUCG UAACGCUAUC GACAAGUGCA   30

(54) INFORMATION FOR SEQ ID NO:53:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AAGGGGAAAC GUUGAGUCCG GUACACCCUG   30

(55) INFORMATION FOR SEQ ID NO:54:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

AGGGUAACGU ACUGGCAAGC UCACCUCAGC   30

(56) INFORMATION FOR SEQ ID NO:55:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GAGGUAACGU ACGACAAGAC CACUCCAACU   30

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(57) INFORMATION FOR SEQ ID NO:56:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

AGGUAACGCU GAGUCAAGUG CACUCGACAU   30

(58) INFORMATION FOR SEQ ID NO:57:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GGGAAACGCU AUCGACGAGU GCACCCGGCA   30

(59) INFORMATION FOR SEQ ID NO:58:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CCGAGGGUAA CGUUGGGUCA AGCACACCUC   30

(60) INFORMATION FOR SEQ ID NO:59:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

UCGGGGUAAC GUAUUGGCAA GGCACCCGAC   30

(61) INFORMATION FOR SEQ ID NO:60:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GGUAACGCUG UGGACAAGUG CACCAGCUGC   30

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(62) INFORMATION FOR SEQ ID NO: 61:

    (i) SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

AGGGUAACGU ACUGGCAAGC UCACCUCAGC   30

(63) INFORMATION FOR SEQ ID NO:62:

    (i) SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

AGGGUAACGU AUAGUCAAGA CACCUCAAGU   30

(64) INFORMATION FOR SEQ ID NO:63:

    (i) SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

GGGUAACGCA UUGGCAAGAC ACCCAGCCCC   30

(65) INFORMATION FOR SEQ ID NO:64:

    (i) SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GAGGAAACGU ACCGUCGAGC CACUCCAUGC   30

(66) INFORMATION FOR SEQ ID NO:65:

    (i) SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

AGGUAACGCU GAGUCAAGUG CACUCGACAU   30

(67) INFORMATION FOR SEQ ID NO:66:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 30 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GGGUAACGUG UGACAAGAUC ACCCAGUUUG   30

(68) INFORMATION FOR SEQ ID NO:67:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 30 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CACAGGGCAA CGCUGCUGAC AAGUGCACCU   30

(69) INFORMATION FOR SEQ ID NO:68:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 30 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

ACGCCAAGUG AGUCAGCAAC AGAGCGUCCG   30

(70) INFORMATION FOR SEQ ID NO:69:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 30 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

CCAGUGAGUC CUGGUAAUCC GCAUCGGGCU   30

(71) INFORMATION FOR SEQ ID NO:70:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 30 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CUUCAGAACG GCAUAGUGGU CGGCCGCGCC   30

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(72) INFORMATION FOR SEQ ID NO:71:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

AGGUCACUGC GUCACCGUAC AUGCCUGGCC   30

(73) INFORMATION FOR SEQ ID NO:72:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

UCCAACGAAC GGCCCUCGUA UUCAGCCACC   30

(74) INFORMATION FOR SEQ ID NO:73:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

ACUGGAACCU GACGUAGUAC AGCGACCCUC   30

(75) INFORMATION FOR SEQ ID NO:74:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 29 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

UCUCGCUGCG CCUACACGGC AUGCCGGGA   29

(76) INFORMATION FOR SEQ ID NO:75:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

GAUCACUGCG CAAUGCCUGC AUACCUGGUC   30

(77) INFORMATION FOR SEQ ID NO:76:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:76:

UCUCGCUGCG CCUACACGGC AUGCCCGGGA   30

(78) INFORMATION FOR SEQ ID NO:77:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:77:

UGACCAGCUG CAUCCGACGA UAUACCCUGG   30

(79) INFORMATION FOR SEQ ID NO:78:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:78:

GGCACACUCC AACGAGGUAA CGUUACGGCG   30

(80) INFORMATION FOR SEQ ID NO:79:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:79:

AGCGGAACGC CACGUAGUAC GCCGACCCUC   30

(81) INFORMATION FOR SEQ ID NO:80:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:80:

ACCCACGCCC GACAACCGAU GAGUUCUCGG   30

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(82) INFORMATION FOR SEQ ID NO:81:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

UGCUUUGAAG UCCUCCCCGC CUCUCGAGGU   30

(83) INFORMATION FOR SEQ ID NO:82:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

AUGCUGAGGA UAUUGUGACC ACUUCGGCGU   30

(84) INFORMATION FOR SEQ ID NO:83:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 29 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

ACCCACGCCC GACAACCGAU GAGCUCGGA   29

(85) INFORMATION FOR SEQ ID NO:84:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

AGUCCGGAUG CCCCACUGGG ACUACAUUGU   30

(86) INFORMATION FOR SEQ ID NO:85:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

AAGUCCGAAU GCCACUGGGA CUACCACUGA   30

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(87) INFORMATION FOR SEQ ID NO:86:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 30 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

ACUCUCACUG CGAUUCGAAA UCAUGCCUGG   30

(88) INFORMATION FOR SEQ ID NO:87:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 30 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

AGGCUGGGUC ACCGACAACU GCCCGCCAGC   30

(89) INFORMATION FOR SEQ ID NO:88:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 30 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

AGCCGCAGGU AACGGACCGG CGAGACCACU   30

(90) INFORMATION FOR SEQ ID NO:89:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 30 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

GCAUGAAGCG GAACUGUAGU ACGCGAUCCA   30

(91) INFORMATION FOR SEQ ID NO:90:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 30 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GGGUAACGUU GUGACAAGUA CACCUGCGUU   30

(92) INFORMATION FOR SEQ ID NO:91:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

GGGUAACGUU GUGACAAGUA CACCUGCGUC   30

(93) INFORMATION FOR SEQ ID NO:92:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

GGGUAACGUU GUGACAAGUA CACCUGCGUC   30

(94) INFORMATION FOR SEQ ID NO:93:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

GGGUAACGUU GUGACAACUA CACCUGCGUC   30

(95) INFORMATION FOR SEQ ID NO:94:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

GGGUAACGUU GUGACAACUA CACCUGCGUC   30

(96) INFORMATION FOR SEQ ID NO:95:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GGGUAACGUU GUGACAACUA CACCUGCGUC   30

(97) INFORMATION FOR SEQ ID NO:96:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

CGUCAGAAGG CAACGUAUAG GCAAGCACAC   30

(98) INFORMATION FOR SEQ ID NO:97:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

GUAGCACUAU CGGCCUAACC CGGUAGCUCC   30

(99) INFORMATION FOR SEQ ID NO:98:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

ACCCGCGGCC UCCGAAGCUA ACCAGGACAC   30

(100) INFORMATION FOR SEQ ID NO:99:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

AGGUCACUGC GUCACCGUAC AUGCCUGGCC   30

(101) INFORMATION FOR SEQ ID NO:100:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

AGGUCACUGC GUCACCGUAC AUGCCUGGCC   30

(102)   INFORMATION FOR SEQ ID NO:101:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

GGCACACUCC AACGAGGUAA CGUUACGGCG   30

(103)   INFORMATION FOR SEQ ID NO:102:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

GGGGCAACGC UACAGACAAG UGCACCCAAC   30

(104)   INFORMATION FOR SEQ ID NO:103:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

GGGGCAACGC UACAGACAAG UGCACCCAAC   30

(105)   INFORMATION FOR SEQ ID NO:104:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

GGGGCAACGC UACAGACAAG UGCACCCAAC   30

(106)   INFORMATION FOR SEQ ID NO:105:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

UGGGUGCUAA CCAGGACACA CCCACGCUGU   30

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(107)   INFORMATION FOR SEQ ID NO:106:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

CCGAGGGUAA CGUUGGGUCA AGCACACCUC   30

(108)   INFORMATION FOR SEQ ID NO:107:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

GGGAAACGCU AUCGACGAGU GCACCCGGCA   30

(109)   INFORMATION FOR SEQ ID NO:108:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

GGGAAACGCU AUCGACGAGU GCACCCGGCA   30

(110)   INFORMATION FOR SEQ ID NO:109:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

ACUCUCACUG CGAUUCGAAA UCAUGCCUGG   30

(111)   INFORMATION FOR SEQ ID NO:110:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

GCAUGAAGCG GAACUGUAGU ACGCGAUCCA   30

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(112)   INFORMATION FOR SEQ ID NO:111:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 30 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GCAUGAAGCG GAACUGUAGU ACGCGAUCCA   30

(113)   INFORMATION FOR SEQ ID NO:112:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 30 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

AGGGUAACGU ACUGGCAAGC UCACCUCAGC   30

(114)   INFORMATION FOR SEQ ID NO:113:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 30 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

AGGGUAACGU ACUGGCAAGC UCACCUCAGC   30

(115)   INFORMATION FOR SEQ ID NO:114:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 30 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

GGUAACGCUG UGGACAAGUG CACCAGCUGC   30

(116)   INFORMATION FOR SEQ ID NO:115:
   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 32 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

UAGCUCGUGA GGCUUUCGUG CUGUUCCGAG CU   32

(117)   INFORMATION FOR SEQ ID NO:116:
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 31 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

UGCAUGUGAG GCGGUAACGC UGUUCCGUGC U   31

(118)   INFORMATION FOR SEQ ID NO:117:
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

UGGUGAGUGA GGCCGAUGCU GUUCCUCGCC GCU   33

(119)   INFORMATION FOR SEQ ID NO:118:
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 35 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

UGACGCGCGA GGUCUUGGUA CUGUUCCGUG GCUCU   35

(120)   INFORMATION FOR SEQ ID NO:119:
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

UCUGGGUGAG ACUUGAAGUC GUUCCCCAGG UCU   33

(121)   INFORMATION FOR SEQ ID NO:120:
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

UCCCGGUGAA GCAUAAUGCU GUUCCUGGGG UCU   33

(122)   INFORMATION FOR SEQ ID NO:121
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid

```
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:


UGGGAGUGAG GUUCCCCGUU CCUCCCGCAC CCU                                    33


 (123)      INFORMATION FOR SEQ ID NO:122:
       (i)  SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:


UAGCGAUGUG AAGUGAUACU GGUCCAUCGU GCU                                    33


 (124)      INFORMATION FOR SEQ ID NO:123:
       (i)  SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 32 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:


UCACAGUGAG CCUUCUGGUG GUCCUGUGUG CU                                     32


 (125)      INFORMATION FOR SEQ ID NO:124:
       (i)  SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:


UUGUUGUGAG UGGUUGAUUC CAUGGUCCAA CCU                                    33


 (126)      INFORMATION FOR SEQ ID NO:125:
       (i)  SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 33 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:


UGCCUGUGAG CUGUUUAGCG GUCCAGGUCG UCU                                    33


 (127)      INFORMATION FOR SEQ ID NO:126:
       (i)  SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 32 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:
```

UCAAGGCGAA GACUUAGUCU GCUCCCUGUG CU   32

(128)   INFORMATION FOR SEQ ID NO:127:
     (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 32 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

UUGCGUCGAA GUUAAUUCUG GUCGAUGCCA CU   32

(129)   INFORMATION FOR SEQ ID NO:128:
     (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 34 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

UUUCAAUGAG GUAUGUAAUG AUGGUCGUGC GCCU   34

(130)   INFORMATION FOR SEQ ID NO:129:
     (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 33 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

UGCGGGAGAG UCUUUUGACG UUGCUCCUGC GCU   33

(131)   INFORMATION FOR SEQ ID NO:130:
     (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 36 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

UCAUGGGAGC CCAUCGAUUC UGGGUGUUGC CUAUGA   36

(132)   INFORMATION FOR SEQ ID NO:131:
     (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 32 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY:' linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

UUGCACAGAG CCAAAUUUGG UGUUGCUGUG CU   32

(133)   INFORMATION FOR SEQ ID NO:132:
     (i)   SEQUENCE CHARACTERISTICS:

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```
        (A)    LENGTH: 33 base pairs
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
        (D)    TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

UGGCCAGAGC UUAAAUUCAA GUGUUGCUGG CCU   33

(134)   INFORMATION FOR SEQ ID NO:133:
    (i)   SEQUENCE CHARACTERISTICS:
```
        (A)    LENGTH: 37 base pairs
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
        (D)    TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

UCAUAGCAGU CCUUGAUACU AUGGAUGGUG GCUAUGA   37

(135)   INFORMATION FOR SEQ ID NO:134:
    (i)   SEQUENCE CHARACTERISTICS:
```
        (A)    LENGTH: 33 base pairs
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
        (D)    TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

UGGAUGCAAG UUAACUCUGG UGGCAUCCGU CCU   33

(136)   INFORMATION FOR SEQ ID NO:135:
    (i)   SEQUENCE CHARACTERISTICS:
```
        (A)    LENGTH: 34 base pairs
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
        (D)    TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

UCAGUGGAGA UUAAGCCUCG CUAGGGGCCG CUAU   34

(137)   INFORMATION FOR SEQ ID NO:136:
    (i)   SEQUENCE CHARACTERISTICS:
```
        (A)    LENGTH: 28 base pairs
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
        (D)    TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

GGUCCGAAGU GCAACGGGGAA AAUGCAC   27

(138)   INFORMATION FOR SEQ ID NO:137:

    (i)   SEQUENCE CHARACTERISTICS:
```
        (A)    LENGTH: 76 base pairs
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
```

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(D)   TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

AGAUGCCUGU CGAGCAUGCU GAGGAUCGAA GUUAGUAGGC UUUGUGUGCU   50
CGUAGCUAAA CAGCUUUGUC GACGGG   76

(139)   INFORMATION FOR SEQ ID NO:138:

     (i) SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 74 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

AGAUGCCUGU CGAGCAUGCU GUACUGGAUC GAAGGUAGUA GGCAGUCACG   50
UAGCUAAACA GCUUUGUCGA CGGG   74

(140)   INFORMATION FOR SEQ ID NO:139:

     (i) SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 74 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

AGAUGCCUGU CGAGCAUGCU GAUAUCACGG AUCGAAGGAA GUAGGCGUGG   50
UAGCUAAACA GCUUUGUCGA CGGG   74

(141)   INFORMATION FOR SEQ ID NO:140:

     (i) SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 75 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

AGAUGCCUGU CGAGCAUGCU GCCUUUCCCG GGUUCGAAGU CAGUAGGCCG   50
GGUAGCUAAA CAGUUUGUCG ACGGG   75

(142)   INFORMATION FOR SEQ ID NO:141:

     (i) SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 75 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

AGAUGCCUGU CGAGCAUGCU GCACCCGGAU CGAAGUUAGU AGGCGUGAGU   50
GUAGCUAAAC AGCUUUGUCG ACGGG   75

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(143)   INFORMATION FOR SEQ ID NO:142:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 76 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

AGAUGCCUGU CGAGCAUGCU GUGUACGGAU CGAAGGUAGU AGGCAGGUUA   50
CGUAGCUAAA CAGCUUUGUC GACGGG   76

(144)   INFORMATION FOR SEQ ID NO:143:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 76 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

AGAUGCCUGU CGAGCAUGCU GCAUCCGGAU CGAAGUUAGU AGGCCGAGGU   50
GGUAGCUAAA CAGCUUUGUC GACGGG   76

(145)   INFORMATION FOR SEQ ID NO:144:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 76 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

AGAUGCCUGU CGAGCAUGCU GAUUGUUGCG GAUCGAAGUG AGUAGGCGCU   50
AGUAGCUAAA CAGCUUUGUC GACGGG   76

(146)   INFORMATION FOR SEQ ID NO:145:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 76 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

AGAUGCCUGU CGAGCAUGCU GUGUACUGGA UCGAAGGUAG UAGGCAGUCA   50
CGUAGCUAAA CAGCUUUGUC GACGGG   76

(147)   INFORMATION FOR SEQ ID NO:146:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 69 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:

```
AGAUGCCUGU CGAGCAUGCU GAUCGAAGUU AGUAGGAGCG UGUGGUAGCU      50
AAACAGCUUU GUCGACGGG                                        69
```

(148)   INFORMATION FOR SEQ ID NO:147:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 81 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:
```
AGAUGCCUGU CGAGCAUGCU GACGCUGGAG UCGGAUCGAA AGGUAAGUAG      50
GCGACUGUAG CUAAACAGCU UUGUCGACGG G                          81
```

(149)   INFORMATION FOR SEQ ID NO:148:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 75 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

```
AGAUGCCUGU CGAGCAUGCU GGGGUCGGAU CGAAAGGUAA GUAGGCGACU      50
GUAGCUAAAC AGCUUUGUCG ACGGG                                 75
```

(150)   INFORMATION FOR SEQ ID NO:149:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 74 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

```
AGAUGCCUGU CGAGCAUGCU GAUAUCACGG AUCGAAAGAG AGUAGGCGUG      50
UAGCUAAACA GCUUUGUCGA CGGG                                  74
```

(151)   INFORMATION FOR SEQ ID NO:150:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 76 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

```
AGAUGCCUGU CGAGCAUGCU GUGUACUGGA UCGAAGGUAG UAGGCAGGCA      50
CGUAGCUAAA CAGCUUUGUC GACGGG                                76
```

(152)   INFORMATION FOR SEQ ID NO:151:

    (i) SEQUENCE CHARACTERISTICS:

-144-

```
        (A)   LENGTH: 75 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

AGAUGCCUGU CGDGCAUGCU GAUAUCACGG AUCGAAGGAA AGUAGGCGUG   50
GUAGCUAAAC AGCUUUGUCG ACGGG   75

(153)   INFORMATION FOR SEQ ID NO:152:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 72 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

AGAUGCCUGU CGAGCAUGCU GGUGCGGCUU UGGGCGCCGU GCUUGGCGUA   50
GCUAAACAGC UUUGUCGACG GG   72

(154)   INFORMATION FOR SEQ ID NO:153:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 71 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

AGAUGCCUGU CGAGCAUGCU GGUGCGGCUU UGGGCGCCGU GCUUACGUAG   50
CUAAACAGCU UUGUCGACGG G   71

(155)   INFORMATION FOR SEQ ID NO:154:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 72 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

AGAUGCCUGU CGAGCAUGCU GGUGCGGCUU UGGGCGCCGU GCUUGACGUA   50
GCUAAACAGC UUUGUCGACG GG   72

(156)   INFORMATION FOR SEQ ID NO:155:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 72 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:
```

AGAUGCCUGU CGAGCAUGCU GGGGCGGCUU UGGGCGCCGU GCUUGACGUA   50
GCUAAACAGC UUUGUCGACG GG   72

   (157)   INFORMATION FOR SEQ ID NO:156:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 79 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:

GGGAGAUGCC UGUCGAGCAU GCUGAGGAUC GAAGUUAGUA GGCUUUGUGU   50
GCUCGUAGCU AAACAGCUUU GUCGACGGG   79

   (158)   INFORMATION FOR SEQ ID NO:157:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 79 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:

GGGAGAUGCC UGUCGAGCAU GCUGCAUCCG GAUCGAAGUU AGUAGGCCGA   50
GGUGGUAGCU AAACAGCUUU GUCGACGGG   79

   (159)   INFORMATION FOR SEQ ID NO:158:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 79 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

GGGAGAUGCC UGUCGAGCAU GCUGAUUGUU GCGGAUCGAA GUGAGUAGGC   50
GCUAGUAGCU AAACAGCUUU GUCGACGGG   79

   (160)   INFORMATION FOR SEQ ID NO:159:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 75 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

GGGAGAUGCC UGUCGAGCAU GCUGGUGCGG CUUUGGGCGC CGUGCUUGAC   50
GUAGCUAAAC AGCUUUGUCG ACGGG   75

WE CLAIM:

1. A method for producing an improved nucleic acid
    ligand from a candidate mixture of nucleic acids,
    said nucleic acid ligand being a ligand of a given
    target comprising:
        a) contacting the candidate mixture with the
    target, wherein nucleic acids having an increased
    affinity to the target relative to the candidate
    mixture may be partitioned from the remainder of
    the candidate mixture;
        b) partitioning the increased affinity
    nucleic acids from the remainder of the candidate
    mixture;
        c) amplifying the increased affinity nucleic
    acids to yield a ligand-enriched mixture of
    nucleic acids;
        d) repeating steps a) - c), as necessary, to
    identify a nucleic acid ligand;
        e) determining the nucleic acid residues of
    the nucleic acid ligand that are necessary for
    binding said target; and
        f) producing said improved nucleic acid
    ligand based on said determination.

2. The method of claim 1 wherein said determination
    includes:
        a) preparing a modified nucleic acid that is
    identical to the nucleic acid ligand except for a
    single residue substitution; and
        b) assessing the binding affinity of the
    modified nucleic acid relative to the nucleic acid
    ligand.

3. The method of claim 1 wherein said determination
    includes:
        a) preparing a modified nucleic acid that is
    identical to the nucleic acid ligand except for
    the absence of one or more terminal residues; and
        b) assessing the binding affinity of the

modified nucleic acid relative to the nucleic acid
ligand.

4.   The method of claim 1 wherein said determination
      includes:
           a)   preparing a modified nucleic acid by
      chemically modifying the nucleic acid ligand; and
           b)   assessing the binding affinity of the
      modified nucleic acid relative to the nucleic acid
      ligand.

5.   The method of claim 1 wherein said determination
      includes:
           a)   chemically modifying said nucleic acid
      ligand in the presence of said target; and
           b)   determining which nucleic acid residues
      are not modified.

6.   A method for producing an improved nucleic acid
      ligand from a candidate mixture of nucleic acids,
      said nucleic acid ligand being a ligand of a given
      target comprising:
           a)   contacting the candidate mixture with the
      target, wherein nucleic acids having an increased
      affinity to the target relative to the candidate
      mixture may be partitioned from the remainder of
      the candidate mixture;
           b)   partitioning the increased affinity
      nucleic acids from the remainder of the candidate
      mixture;
           c)   amplifying the increased affinity nucleic
      acids to yield a ligand-enriched mixture of
      nucleic acids;
           d)   repeating steps a) - c) as necessary to
      identify said nucleic acid ligands;
           e)   determining the three-dimensional
      structure of said nucleic acid ligand; and
           f)   producing said improved nucleic acid
      ligand based on said determination.

7.   The method of claim 6 wherein said determination
     includes:
          a)   denaturing the nucleic acid ligand;
          b)   chemically modifying denatured and
     nondenatured nucleic acid ligand; and
          c)   determining which nucleic acid residues
     are modified in the denatured nucleic acid ligand
     that are not modified in the nondenatured nucleic
     acid ligand.

8.   The method of claim 6 wherein said determination
     includes:
          a)   preforming covariance analysis on said
     nucleic acid ligand.

9.   A method for producing an improved nucleic acid
     ligand to a given target from a plurality of
     nucleic acid ligands comprising:
          a)   determining the nucleic acid residues of
     said nucleic acid ligands that are necessary for
     binding said target; and
          b)   determining the three dimensional
     structure of said nucleic acid ligands.

10. A method for identifying an extended nucleic acid
     ligand from a candidate mixture of nucleic acids,
     said nucleic acid ligand being a ligand of a given
     target comprising:
          a)   contacting the candidate mixture with the
     target, wherein nucleic acids having an increased
     affinity to the target relative to the candidate
     mixture may be partitioned from the remainder of
     the candidate mixture;
          b)   partitioning the increased affinity
     nucleic acids from the remainder of the candidate
     mixture;
          c)   amplifying the increased affinity nucleic
     acids to yield a ligand-enriched mixture of
     nucleic acids;
          d)   repeating steps a) - c), as necessary, to

identify said nucleic acid ligand;

e)   creating a second candidate mixture
comprised of nucleic acids having a fixed region
and a randomized region, wherein said fixed region
corresponds to said nucleic acid ligand identified
in d) above;

f)   contacting the second candidate mixture
with the target, wherein nucleic acids having an
increased affinity to the target relative to the
candidate mixture may be partitioned from the
remainder of the candidate mixture;

g)   amplifying the increased affinity nucleic
acids to yield a ligand-enriched mixture of
nucleic acids; and

h)   repeating steps e) - g), as necessary, to
identify said nucleic acid ligand.

11.   A non-naturally occurring nucleic acid ligand to
the HIV-RT protein.

12.   The nucleic acid ligand of claim 11 produced
according to the method of claim 1.

13.   The nucleic acid ligand of claim 12 comprising the
additional steps of
d)   repeating steps a) -c), as necessary, to
identify a nucleic acid ligand;
e)   determining the nucleic acid residues of the
nucleic acid ligand that are necessary for binding
said HIV-RT; and
f)   producing said improved nucleic acid ligand
based on said determination.

14.   The nucleic acid ligand of claim 11 produced
according to the method of claim 2.

15.   The nucleic acid ligand of claim 11 produced
according to the method of claim 3.

16.   The nucleic acid ligand of claim 11 produced

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according to the method of claim 4.
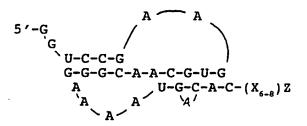
17.   The nucleic acid ligand of claim 11 produced
      according to the method of claim 5.

18.   The nucleic acid ligand of claim 11 having the
      sequence:

```
                  ,X — X ,
               A /        \ X
5-U-C-C-S'               \
    G-G-S'-A-X -X -X
    G              X'-X'-X'-3'
     \             A
      A      A'
       \  X /
```

      wherein X-X' indicates a preferred base-pair.

19.   The nucleic acid ligand of claim 11 having a
      sequence that is substantially homologous to and
      has substantially the same ability to bind HIV-RT
      as the sequence:

```
                  , X — X ,
               A /         \ X
5'-U-C-C-S'                /
     G-G-S'-A-X -X -X
     G             ,X'-X'-X'-3'
      \            A
       A     A'
        \ X /
```
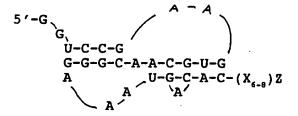
      wherein X-X' indicates a preferred base-pair.

20.   The nucleic acid of claim 11 identified according
      to the method of claim 10.

21.   The nucleic acid ligand of claim 20 having the
      sequence:

```
                   A — A
        5'-G     /        \
          'G    /          )
            \ U-C-C-G     /
             G-G-G-C-A-A-C-G-U-G
             A           ,U-G-C-A-C-(X_{6-8})Z
              'A    A /    'A/
                \ A /
```

wherein Z is selected from the group consisting of
the sequences set forth in Figure 9 (SEQ ID NO:115-135)
[10].
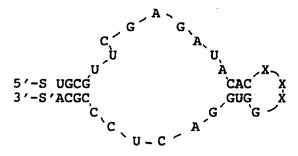
22.   The nucleic acid ligand of claim 20 having a
       sequence that is substantially homologous to and
       has substantially the same ability to bind HIV-RT
       as the sequence:

```
5'-G                          A ⁻ A
     G                      /          \
      U-C-C-G             /             )
      G-G-G-C-A-A-C-G-U-G
      A              U-G-C-A-C-(X₆₋₈)Z
        \         A       A
          A     A
```
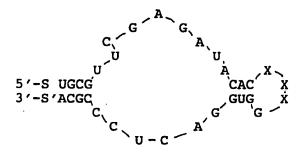
       wherein Z is selected from the group consisting of
the sequences set forth in Figure 9 (SEQ ID NO:115-
135).

23.   The nucleic acid ligand of claim 21 wherein Z is
       selected from the group consisting of: extension
       motif I and extension motif II, each as set forth
       in Figure 9 (SEQ ID NO:115-135).

24.   The nucleic acid ligand of claim 22 wherein Z is
       selected from the group consisting of: extension
       motif I and extension motif II, each as set forth
       in Figure 9 (SEQ ID NO:115-135).

25.   A non-naturally occurring nucleic acid ligand of
       the HIV-1 Rev protein.

26.   The nucleic acid ligand of claim 25 produced
       according to the method of claim 1.

27.   The nucleic acid ligand of claim 25 produced
       according to the method of claim 2.

28.   The nucleic acid ligand of claim 25 produced
       according to the method of claim 3.

29. The nucleic acid ligand of claim 25 produced
     according to the method of claim 4.

30. The nucleic acid ligand of claim 25 produced
     according to the method of claim 5.

31. The nucleic acid ligand of claim 25 having the
     sequence:

```
                   A
              G        G
          C            A
            U          A  U
          U               A
5'-S UGCG               CAC    X
3'-S'ACGC               GUG    X
          C           G    G
            C       A
              U - C
```

32. The nucleic acid ligand of claim 25 having a
     sequence that is substantially homologous to and
     has substantially the same ability to bind the
     HIV-1 Rev protein as the sequence:

```
                   A
              G        G
          C            A
            U          A  U
          U               A
5'-S UGCG               CAC    X
3'-S'ACGC               GUG    X
          C           G    G
            C       A
              U - C
```


33. A method of producing an improved ligand to a
     given target comprising:
          (a) preparing a candidate mixture of nucleic
     acids;
          (b) contacting the candidate mixture with
     the target, where nucleic acids having an
     increased affinity to the target relative to the
     candidate mixture may be partitioned from the
     remainder of the candidate mixture;
          (c) partitioning the increased affinity

nucleic acids from the remainder of the candidate
mixture;

      (d) amplifying the increased affinity
nucleic acids to yield a ligand-enriched mixture
of nucleic acids;

      (e) repeating steps (b)-(d), as necessary,
to identify a nucleic acid ligand;

      (f) determining the nucleic acid residues of
the nucleic acid ligand that are necessary for
binding said target;

      (g) determining the three-dimensional
structure of said nucleic acid ligand; and

      (h) designing said improved ligand based on
said determinations.

34. A method for identifying nucleic acid ligands to
the HIV-1 tat protein comprising:

      a) preparing a candidate mixture of nucleic
acids,

      b) partitioning between members of said
candidate mixture on the basis of affinity to said
protein; and

      c) amplifying selected molecules of the
candidate mixture with a relatively higher
affinity for said protein to yield a mixture of
nucleic acids enriched for sequences with a
relatively higher affinity to the protein.

35. The method of claim 34 further comprising
      d) repeating steps b) and c).

36. The method of claim 34 wherein said candidate
mixture of nucleic acids is comprised of single
stranded nucleic acids.

37. The method of claim 36 wherein said candidate
mixture is comprised of RNA.

38. A nucleic acid ligand to the HIV-1 tat protein
identified according to the method of claim 34.

39.   The nucleic acid ligand of claim 38 being a single
      stranded nucleic acid.

40.   The nucleic acid ligand of claim 38 being a
      single stranded RNA sequence.

41.   A purified and isolated non-naturally occurring
      nucleic acid ligand to the HIV-1 tat protein.

42.   The nucleic acid ligand of claim 41 wherein said
      ligand is selected from the group consisting of
      the sequences set forth in Figure 26.

43.   The nucleic acid ligand of claim 41 wherein said
      ligand is substantially homologous to and has
      substantially the same ability to bind the tat
      protein as a ligand selected from the group
      consisting of the sequences set forth in Figure
      26.

44.   The nucleic acid ligand of claim 41 wherein said
      ligand is selected from the group consisting of:
      motif I, motif II and motif III, as shown in
      Figure 27.

45.   The nucleic acid ligand of claim 41 wherein said
      ligand is substantially homologous to and has
      substantially the same ability to bind the tat
      protein as a ligand selected from the group
      consisting of: motif I, motif II and motif III,
      as shown in Figure 27.

46.   The nucleic acid ligand of claim 41 wherein said
      ligand has been chemically modified at the ribose
      and/or phosphate and/or base positions.

47.   The nucleic acid ligand of claim 41 wherein said
      ligand has substantially the same structure and
      substantially the same ability to bind the tat
      protein as a ligand selected from the group

consisting of the sequences set forth in Figure
26.

48. The nucleic acid ligand of claim 41 wherein said
     ligand has substantially the same structure and
     substantially the same ability to bind the <u>tat</u>
     protein as a ligand selected from the group
     consisting of: motif I, motif II and motif III,
     as shown in Figure 27.

49. A method for identifying nucleic acid ligands to
     thrombin comprising:
     a)   preparing a candidate mixture of RNA nucleic
     acids;
     b)   contacting the candidate mixture with
     thrombin, wherein nucleic acids having an
     increased affinity to thrombin may be partitioned
     from the remainder of the candidate mixture;
     c) partitioning between members of said candidate
     mixture on the basis of affinity to thrombin; and
     d) amplifying selected molecules of the candidate
     mixture with a relatively higher affinity for
     thrombin to yield a mixture of nucleic acids
     enriched for sequences with a relatively higher
     affinity to the protein.

50. The method of claim 49 further comprising
     e)   repeating steps b), c) and d).

51. The method of claim 49 wherein said candidate
     mixture of RNA nucleic acids is comprised of
     single stranded nucleic acids.

52. A RNA nucleic acid ligand to thrombin identified
     according to the method of claim 49.

53. The nucleic acid ligand of claim 52 being a single
     stranded nucleic acid.

54. A purified and isolated non-naturally occurring

RNA ligand to thrombin.

55. The RNA ligand of claim 54 wherein said ligand is
    selected from the group consisting of the
    sequences set forth in Figure 29 (SEQ ID NO:137-
    154).

56. The RNA ligand of claim 54 wherein said ligand is
    substantially homologous to and has substantially
    the same ability to bind thrombin as a ligand
    selected from the group consisting of the
    sequences set forth in Figure 29 (SEQ ID NO:137-
    154).

57. The RNA ligand of claim 54 wherein said ligand has
    been chemically modified at the ribose and/or
    phosphate and/or base positions.

58. The RNA ligand of claim 54 wherein said ligand has
    substantially the same structure and substantially
    the same ability to bind thrombin as the sequences
    set forth in Figure 29 (SEQ ID NO:137-154).

59. The RNA ligand of claim 54 comprised of the RNA
    sequence (SEQ ID NO:9):
            5'-GGAUCGAAG(N)$_2$AGUAGGC-3'

60. The RNA ligand of claim 6 comprised of the RNA
    sequence (SEQ ID NO:10):
            5'-GCGGCUUUGGGCGCCGUGCUU-3'

61. The RNA ligand of claim 54 wherein said ligand is
    substantially homologous to and has substantially
    the same ability to bind thrombin as the ligand of
    claim 59.

62. The RNA ligand of claim 54 wherein said ligand has
    substantially the same structure and substantially
    the same ability to bind thrombin as the ligand of
    claim 59.

63. The RNA ligand of claim 54 wherein said ligand is substantially homologous to and has substantially the same ability to bind thrombin as the ligand of claim 60.

64. The RNA ligand of claim 54 wherein said ligand has substantially the same structure and substantially the same ability to bind thrombin as the ligand of claim 60.

65. The RNA ligand of claim 54 prepared according to the method of claim 49.

66. A method for identifying nucleic acid ligands to basic fibrobast growth factor (bFGF) comprising:
   a) preparing a candidate mixture of RNA nucleic acids;
   b) contacting the candidate mixture with bFGF, wherein nucleic acids having an increased affinity to bFGF may be partitioned from the remainder of the candidate mixture;
   c) partitioning between members of said candidate mixture on the basis of affinity to bFGF; and
   d) amplifying selected molecules of the candidate mixture with a relatively higher affinity for bFGF to yield a mixture of nucleic acids enriched for sequences with a relatively higher affinity to bFGF.

67. The method of claim 66 further comprising
   e) repeating steps b), c) and d).

68. The method of claim 66 wherein said candidate mixture of RNA nucleic acids is comprised of single stranded nucleic acids.

69. A RNA nucleic acid ligand to bFGF identified according to the method of claim 66.

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70. The nucleic acid ligand of claim 69 being a single
     stranded nucleic acid.

71. A purified and isolated non-naturally occurring
     RNA ligand to bFGF.

72. The RNA ligand of claim 71 wherein said ligand is
     selected from the group consisting of the
     sequences set forth in Tables II and III (SEQ ID
     NO:28-67).

73. The RNA ligand of claim 71 wherein said ligand is
     substantially homologous to and has substantially
     the same ability to bind bFGF as a ligand selected
     from the group consisting of the sequences set
     forth in Tables II and III. (SEQ ID NO:28-67).

74. The RNA ligand of claim 71 wherein said ligand has
     been chemically modified at the ribose and/or
     phosphate and/or base positions.

75. The RNA ligand of claim 71 wherein said ligand has
     substantially the same structure and substantially
     the same ability to bind bFGF as the sequences set
     forth in Tables II, III, and IV, (SEQ ID NO:28-
     89).

76. The RNA ligand of claim 71 comprised of the RNA
     sequence (SEQ ID NO:27):
                   5'-CUAACCNGG-3'

77. The RNA ligand of claim 71 comprised of the RNA
     sequence (SEQ ID NO:43):
                   5'-RRGGHAACGYWNNGDCAAGNNCACYY-3'
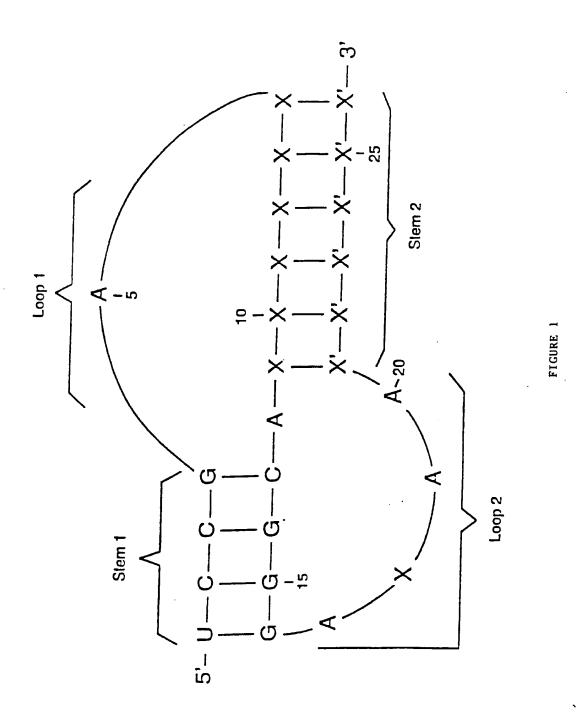
78. The RNA ligand of claim 71 wherein said ligand is
     substantially homologous to and has substantially
     the same ability to bind bFGF as the ligand of
     claim 76.

79. The RNA ligand of claim 71 wherein said ligand has substantially the same structure and substantially the same ability to bind bFGF as the ligand of claim 76.

80. The RNA ligand of claim 71 wherein said ligand is substantially homologous to and has substantially the same ability to bind bFGF as the ligand of claim 77.

81. The RNA ligand of claim 71 wherein said ligand has substantially the same structure and substantially the same ability to bind bFGF as the ligand of claim 77.

82. The RNA ligand of claim 71 wherein said ligand is an inhibitor of bFGF.

83. The RNA ligand of claim 71 wherein said ligand is selected from the group consisting of the sequences set forth in Table II (SEQ ID NO:27-42).

FIGURE 1

FIGURE 2

FIGURE 3

Reactivity of the bases of HIV-RT pseudoknot ligand B to chemical modification under native conditions

FIGURE 4

FIGURE 5

Chemical modification Interference of the
binding of pseudoknot ligand B to HIV-RT

FIGURE 6

FIGURE 7

● : denotes a 2' O-methyl instead of an OH,
    denoted by (O), at this position on the ribose
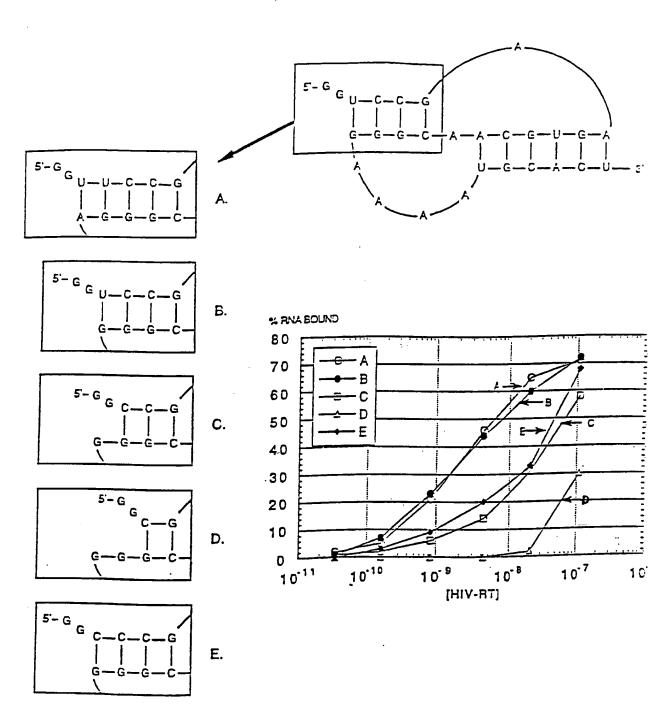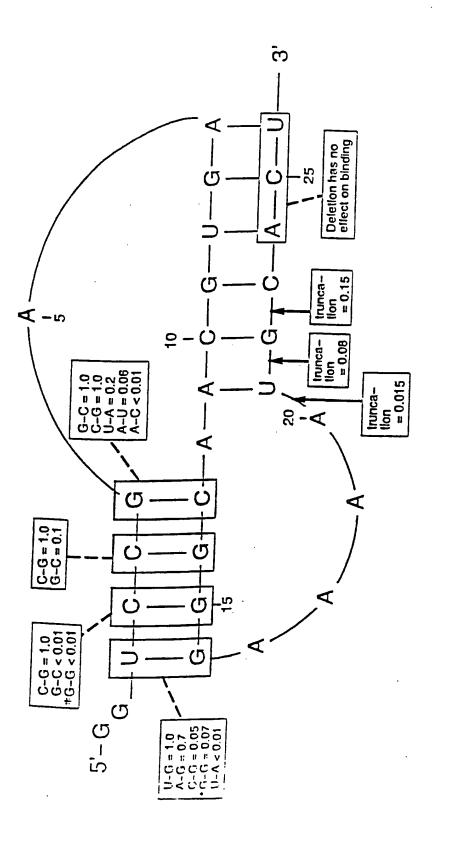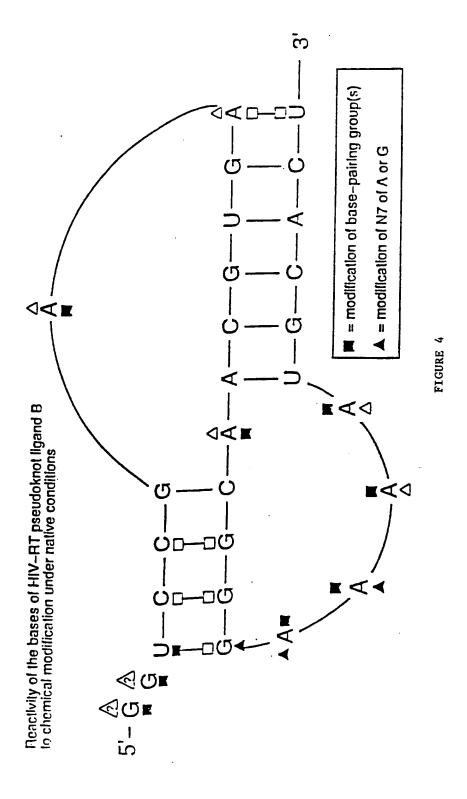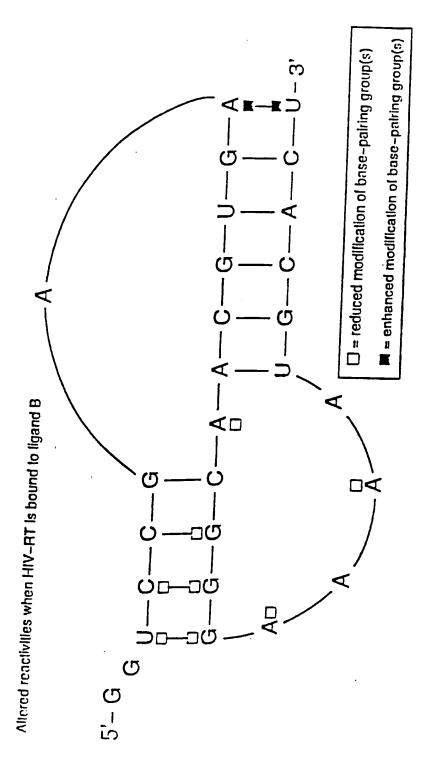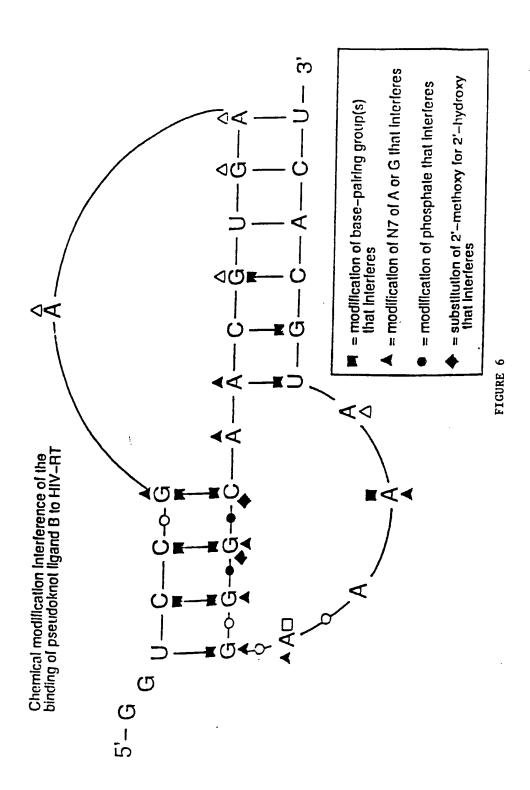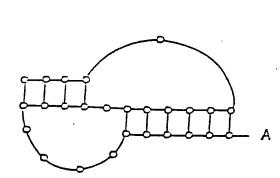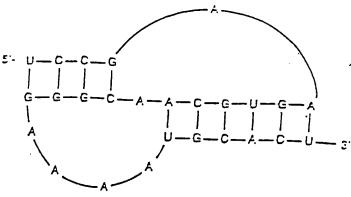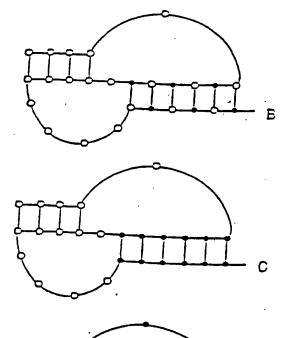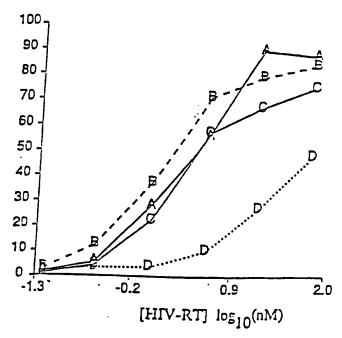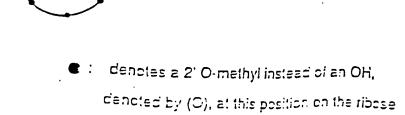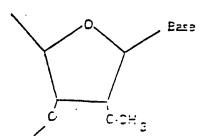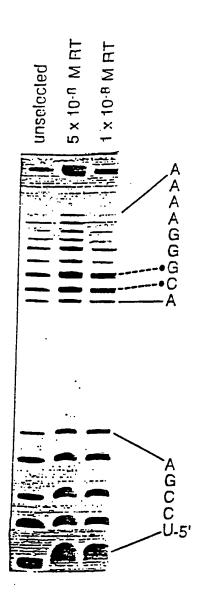
Selection of 2'-methoxy vs.
2'-OH within the HIV-RT
pseudoknot ligand

FIGURE 8

FIGURE 9

Pseudoknot extension sequences

Starting material
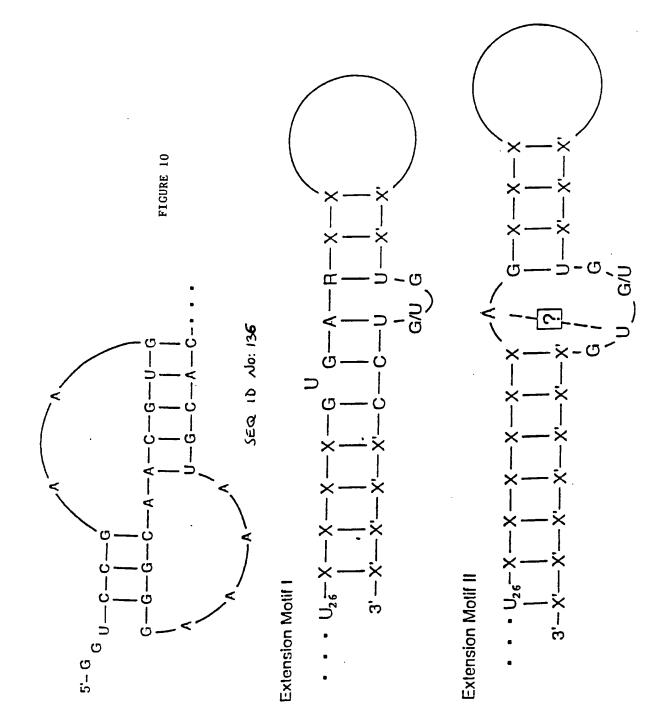
5'-GGUCCGAAGUGCAACGGGAAAUGCACU-[30n]-
                    CUAUGAAGAAUUUUAUAUCUCUAUUGAAAC-3'

Extension Motif I

Isolate number   SEQ ID NO:

3,22,29   uAGCUCPUGAGGCUUU—CGUGCPGUUCQGAGCu   115

14   uGCAUPUGAGGCGGU—AACGCPGUUCQGUGCu   116

20   uGGUGAPUGAGGCCG——AUGCPGUUCQUCGCCGCu   117

4   uGACGCPCGAGGUCUU—GGUACPGUUCQGUGGCUCu   118

30   uCUGGPUGAGACUUG——AAGUPGUUCQCCAGGUCu   119

38   uCCCGPUGAAGCAUA——AUGCPGUUCQUGGGGUCu   120

39   uGGGAPUGAGGUU————CCCPGUUCQUCCCGCACCCu   121

2,6,9   uAGCGAUPUGAAGUGA————UACUPGGUCQAUCGUGCu   122

13,26   uCACAPUGAGCUU————CUGGPGGUCQUGUGUGCu   123

7   uUGUUPUGAGUGGUUGAUUCCAUGGUCQAACcu   124

35   uGCCUPUGAGCUGU——UUAGCPGGUCQAGGUCGUcu   125

24   uCAAGPCGAAGACUU—AGUCPGCUCQCUGUGCu   126

- -

8   uUGCGPCGAAGUUAA————UUCPGGUCGAUGCCAcu   127

40   uUUCAPUGAGGUAUG-UAAUGAUGGUCGUGCGCcu   128

Extension Motif II

1   uGCGGGAGAGUCPUU————UUGACGUUGCUPCUGCGcu   129

17   uCAUGGGAGCCCAUCGA-UUCUGPGUGUUGCCuauga   130

23,27   uUGCACAGAGCCAAA————UUUPGUGUUGCUGUGcu   131

18,34   uGGCCAGAGCUPAAA————UUCAPGUGUUGCUGGCcu   132

19   uCAUAGCPGUCPUUGAUACUAUGPAUGGUGGCuauga   133

37   uGGAUGCAAGUUAA————CPCUGGUGGCAUCCGUCcu   134

_____

11   uCAUUGGAAGAUUAACCCUGGCUAGGGCUUCCUAu   135

FIGURE 10

FIGURE 11

Figure 12

FIGURE 13
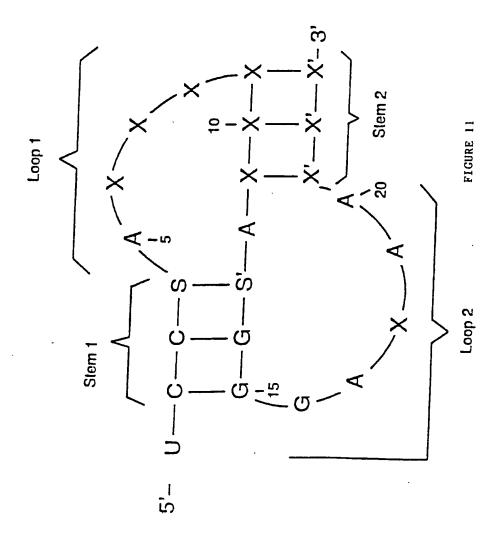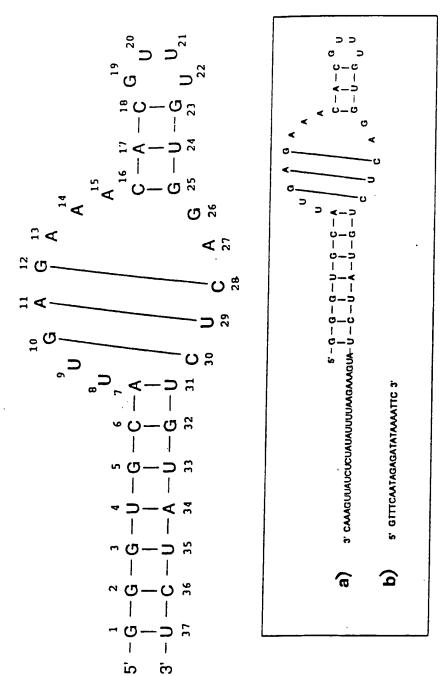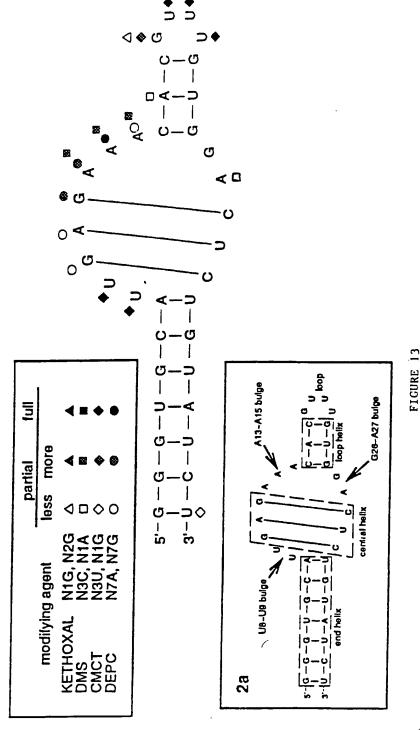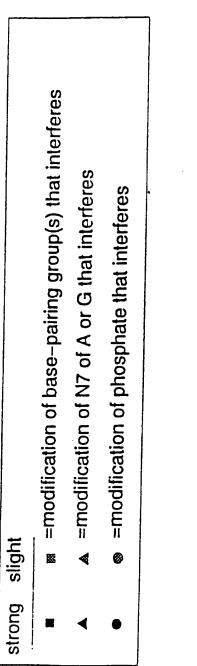
FIGURE 14

Chemical modification interference of ligand binding to Rev

strong   slight

■   ▦   =modification of base–pairing group(s) that interferes

▲   ▲   =modification of N7 of A or G that interferes

●   ●   =modification of phosphate that interferes
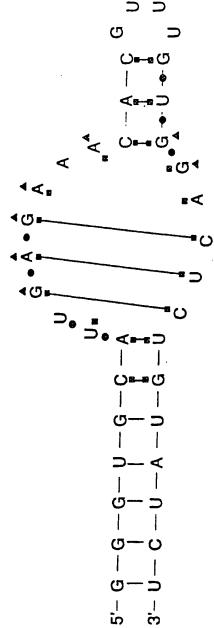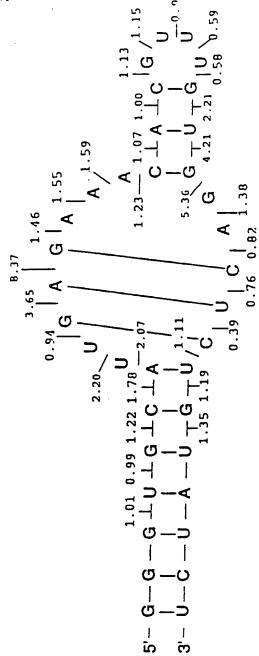
FIGURE 15

Inhibition of Rev binding by phosphate alkylation

(data normalized to A17 3' phosphate)
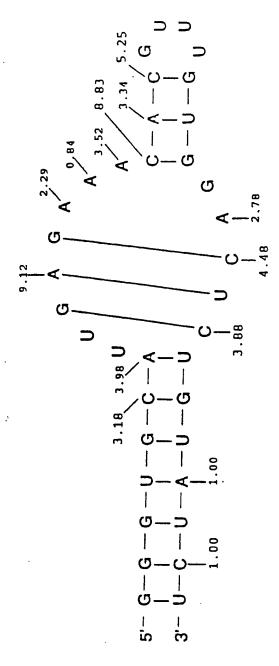
FIGURE 16

Inhibition of Rev binding by DMS modification of N3C & N1A

(data normalized to C36; A34)

FIGURE 17

Inhibition of Rev binding by kethoxal modification of N1G & N2G

(data normalized to G5)

FIGURE 18
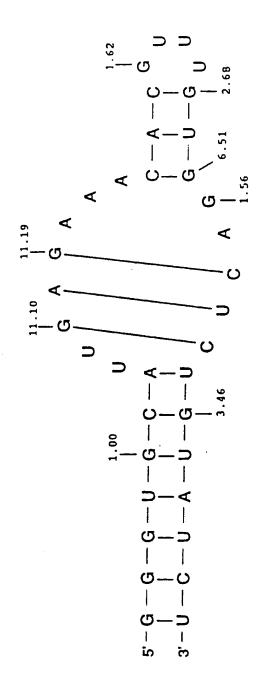
Inhibition of Rev binding by CMCT modification of N3U & N1G
(data normalized to U38)
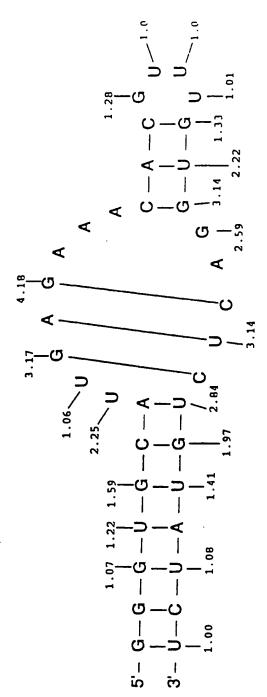
FIGURE 19

Inhibition of Rev binding by DEPC modification of N7A & N7G

(data normalized to G19; A34)
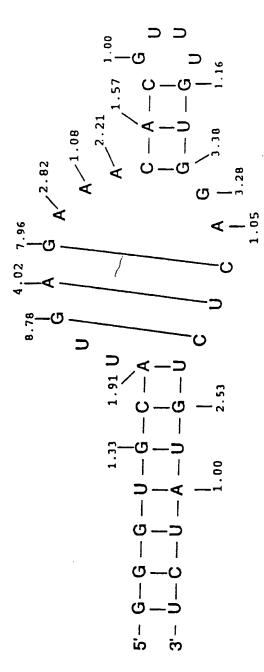
FIGURE 20

Altered chemical reactivities when ligand is bound to Rev

▷ = reduced modification of base

◀ = enhanced modification of base
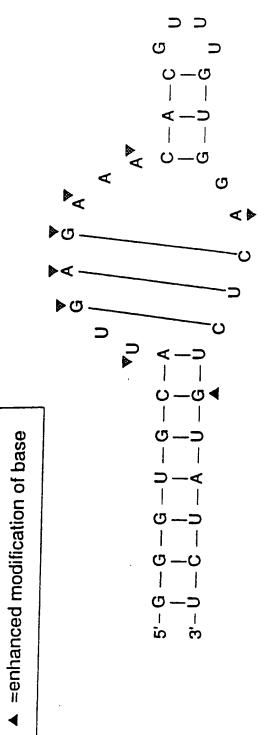
FIGURE 21

5'-GGGACUAUUGAUGGCCUUCCGACC-6a-CACACAGAGGUAAAGAGGAUCCGGG-3'

Biased ligand sequences

6a   GGGUGCAUUGAGAAACACGU-UUGUGGACUCUGUAUCU

1   UGGUGCGUUGAGAAACAGGU-UUUUGGACUCCGUACCa

2   GUAUGCAUUGAGAGACACAC-UUGUGGACUCUGCAUCC

3   AGAUGGAUUGAGAAACACUA-UUAUGGACUCUCCAUCG

4   AGCUUCGUCGAGAUACACGU-UGAUGGACUCCGAAGCA

5   UCGUACGUUGAGAAACAAGU-UUAUGGACUCCGUACCU

6   UCGAUCGUUGAGAUACACGC-UAGUGGACUCCGAAACU

8   UACUGCAUCGAGAUACACGU-UUGUGGACUCUGCACAU

9   UGAUACGUUGAGAAACACAA-UGCUGGACUCCGCAUCC

10   GCCUGCAUUGAGAAACAGGA-UUCUGGACUCUGCCACU

12   CGCUAUGUUGAGAAACACUU-UGCUGGACUCCGUAGCU

13   UACUGCAUCGAGAAACACGU-AAGUG-ACUCUGCAUCC

15   CGGUACGUCGAGAUACACGA-AGAUGGACUCCGUAUCG

17   AACUCCAUCGAGAAACACGA-UAGUGGACUCUGGAGCU

18   GGAGACGUCGAGAAACACGU-UUGUGGACUCCGUCUCU

21   AGCUACAUCGAGAAACAAGA-UUUUGGACUCUGUAGCG

23   AAGUGCAUUGAGAUACAAAU-GAUUGGACUCUGCAcac

24   UGCUACGUUGAGAUACACGU-UGAUGCACUCCGUAGCU

25   AGCUACGUUGAGAUACACGUUACGUGG-CUCCGUAGCC

27   GAGUGGCUCGAGAAACAGGU-UGCUGGACUCGCCACAU

28   UCGUGCGUCGAGCAACACGU-UGAUGGACUCCGCACAG

29   GGCACCGUUGAGAAACACAU-GCGUGGACUCCGUGCCC

30   UCCUGCAUUGAGAAACAGUG-AUCUGGACUCUGCAACU

31   cUGUGGAUUGAGCAACACGU-GAGUGGACUCUCCACAU

32   CCGUGCGUUGAGACACACAC-CGAUGGACUCCGCAUGU

FIGURE 21 CONT.

33   AGCUGCAUCGAGAUACACGA-UUGUGGACUCUGCAGCC

35   AGAUUCGUUGAGAAACACAU-GGGUGGACUCUCCCGCUA

36   AGAUGGAUUGAGAAACACGU-UCGUGGACUCUCCAACU

37   GACUGCAUCGAGAAACACUG-AUGUGGCCUCCGCACGG

38   AGCUACGUUGAGAAACAGUA-UAAUGGACUCCGUAGCU

40   GAGUGCGUCGAGAAACACAU-UUGUGGACUCCGCACAC

42   UCGUACGUUGAGAAACACGC-UAGUGGACUCCGUAUGU

43   AGAUACGUUGAGAGACACGC-ACGUGGACUCCGUAUCU

44   AGGAUCACAGAGAAACACCGUGGGUGG-CUCCCUCUAU

45   GUGCGCAUCGAGAAACACGU-UGAUGGACUCUGCAUGCAC

47   GAGAGGAUCGAGAAACACGU-AUGUGGACUCUCCAUCU

48   GGAUGGAUUGAGACACACGU-AUGUGGACUCUCCAUCA

49   UCGGGCAUUGAGAUACACGU-AGAUGGACUCUGUCUCA

50   UGGACCGUAGAGAAACACGUUUGAUGG-CUCCCUCUGU

FIGURE 22

A.

Distribution of nucleotides found in specific positions within the Rev ligand 6a biased sequence.

6a wt	G	G	G	U	G	G	A	U	A	G	A	G	A	A	A	A	C	A	C	A	C
A	10	19	16	30	20	31	18	37	22	38	38	36	25	38	38	38	30	38	38	38	30
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	12	8	8	5	11	0	18	0	2	0	0	36	25	0	0	0	3	38	38	0	3
U	3	8	14	1	3	31	1	1	14	38	38	2	2	38	38	0	30	0	0	38	30
bp	10	19	16	2	20	4	19	0	0	0	0	0	2	0	0	38	5	0	0	0	5
	16	20	26	30	4	1	0	37	22	38	38	38	9	38	38	38	26	38	38	38	26

B.

Frequency of nucleotides found at each position.

6a wt	G	G	G	U	G	G	A	U	A	G	A	G	A	A	A	A	C	A	C	A	C
A	.26	.50	.42	.79	.53	.82	.47	.97	.58	1.00	1.00	.95	.66	1.00	1.00	1.00	.79	1.00	1.00	1.00	.79
C	.00	.03	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00
G	.32	.21	.21	.13	.29	.00	.47	.00	.05	.00	.00	.95	.66	.00	.00	.00	.08	1.00	1.00	.00	.08
U	.08	.21	.37	.03	.08	.82	.03	.03	.37	1.00	1.00	.05	.05	1.00	1.00	.00	.79	.00	.00	1.00	.79
bp	.26	.50	.42	.05	.53	.11	.50	.00	.00	.00	.00	.00	.24	.00	.00	1.00	.13	.00	.00	.00	.13
	.42	.53	.68	.79	.11	.03	.00	.97	.58	1.00	1.00	1.00	.00	1.00	1.00	1.00	.68	1.00	1.00	1.00	.68
			.82	.87	.92	.92															

C.

Difference between observed frequency from that expected based on input distribution bias during original template synthesis.

6a wt	G	G	G	U	G	G	A	U	A	G	A	G	A	A	A	A	C	A	C	A	C
A	-.37	-.13	-.21	.16	-.10	.19	-.16	.34	-.05	.37	.37	.32	.03	.37	.37	.37	.16	.37	.37	.37	.16
C	.19	.09	.09	.01	-.16	-.13	-.16	-.13	-.07	-.13	-.13	.32	.03	-.13	-.13	-.13	-.05	.37	.37	-.13	-.05
G	-.05	.09	.24	-.09	-.05	-.19	-.10	-.10	.24	.37	.37	-.07	-.07	.37	.37	-.13	.16	-.13	-.13	.37	.16
U	-.37	-.13	-.21	-.07	-.37	-.02	.38	-.13	-.13	-.13	-.13	-.13	-.07	-.13	-.13	.37	.01	-.13	-.13	-.13	.01
	.38	-.07	-.13	.16	-.02	-.10	-.13	.34	-.05	-.13	-.13	.11	-.13	-.13	-.13	-.13	-.13	-.13	-.13	-.13	-.13

FIGURE 22 CONT.

A

Distribution of nucleotides (continued).

B

Frequency of nucleotides found at each position (continued).

C

Difference between observed and expected frequency (continued).

FIGURE 23

Distribution of base-pairs at specific positions within the Rev ligand 6a biased sequence.

Ga	G1/U37	G2/C36	G3/U35	U4/A34	G5/U33	C6/G32	A7/U31	C16/G25	A17/U24	C18/G23	G19/U22
WT	2	16	8	28	2	28	16	38	38	19	9
AU	6	0	6	0	10	0	16	0	38	0	2
UA	2	1	0	28	2	0	0	0	0	2	1
CC	5	16	7	2	18	6	18	0	0	3	3
CG	1	2	8	0	1	28	1	38	0	19	1
CU	1	1	8	0	2	0	1	0	0	1	9
UG	4	1	0	2	0	1	0	0	0	0	0
AC	3	3	0	0	1	0	2	0	0	0	1
CA	1	1	2	0	0	0	0	0	0	9	0
other	16	13	7	8	2	3	0	0	0	4	14

$\mathcal{A}$

Frequency of base-pairings found at each position.

6a	G1/U37	G2/C36	G3/U35	U4/A34	G5/U33	C6/G32	A7/U31	C16/G25	A17/U24	C18/G23	G19/U22
WT	.05	.42	.21	.74	.05	.74	.42	1.00	1.00	.50	.24
AU	.16	.00	.16	.00	.26	.00	.42	.00	1.00	.00	.05
UA	.05	.03	.00	.74	.05	.00	.00	.00	.00	.05	.03
CC	.13	.42	.18	.05	.47	.16	.47	.00	.00	.08	.08
CG	.03	.05	.21	.00	.03	.74	.03	1.00	.00	.50	.03
CU	.03	.03	.21	.00	.05	.00	.03	.00	.00	.03	.24
UG	.11	.03	.00	.05	.00	.03	.00	.00	.00	.00	.00
AC	.08	.08	.00	.00	.03	.00	.05	.00	.00	.00	.03
CA	.03	.03	.05	.00	.00	.00	.00	.00	.00	.24	.00
other	.42	.34	.18	.21	.05	.08	.00	.00	.00	.11	.37

$\mathcal{B}$

FIGURE 23 CONT.

Difference between observed frequency (of base pairs) from that expected based on input distribution bias during original template synthesis.

6a	G1/U37	G2/C36	G3/U35	U4/A34	G5/U33	C6/G32	A7/U31		C16/G25	A17/U24	C18/G23	G19/U22
WT	-.34	.03	-.18	.35	-.34	.35	.03			.61	.11	-.15
AU	.08	-.02	.08	-.02	.19	-.02	.03		.61	.61	-.02	-.03
UA	.04	.01	-.02	.35	.04	-.02	-.02		-.02	-.02	.04	.01
CC	.05	.03	.11	.04	.40	.14	.46		-.02	-.02	.06	.00
CG	.01	.04	.19	-.02	.01	.35	.01		-.02	-.02	.11	.01
CU	-.34	-.05	-.18	-.02	-.34	-.02	-.05		.61	-.08	.01	-.15
UG	.09	.01	-.02	-.08	-.02	-.05	-.02		-.02	-.02	-.08	-.02
AC	.06	.00	-.02	.04	.01	-.02	-.03		-.08	-.08	-.02	.01
CA	.01	.01	.04	-.08	-.02	-.08	-.02		-.02	-.02	.16	-.02
other	.17	.09	-.07	-.04	-.20	-.17	-.25		-.25	-.25	-.15	-.12

Motif I consensus

Ligand 6a sequence

Secondary Consensus derived from SELEX with
biased randomization

TAR RNA
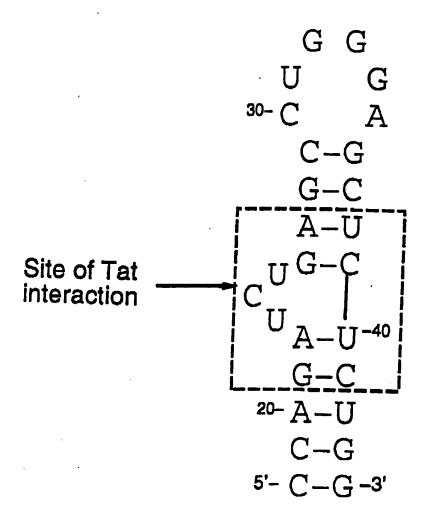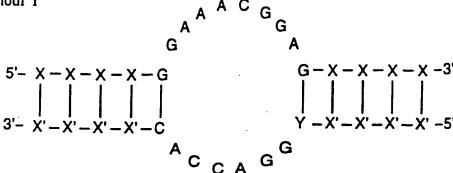
FIGURE 25

Tat Ligand Sequences
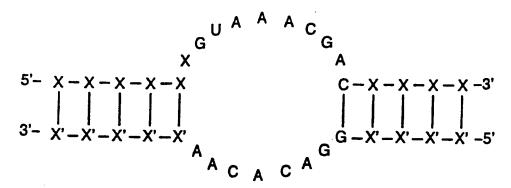
Isolate   Tat ligand
Number   motif

4,12,16   aaGCCUCAGUAAGGCAACGGAAUCCGCA———AGAGGAUGGACCACuucgacaug

7,9   gcucaaCACGAAGGAAACGGAGGGAAUCUU–GAAGAACCCGGACCACuucgacaug

5   cucaaGCGGAAUGGAAACGGAGCCAUCAAC—AAGCUGGCGGACCACAuucgacau

26   ucaaCAAACGAAGGAAACGGAUGACCCAA——GCAGGUCAGGACCACuucgacaug   I

19   cgcucaaACGAAGGAAACGGAUGCGGACAUAUGUGCCGCAGGACCACuucgacaug

21   aaACACAUCGAAGGUAACGGAGCGAAAA———GAACGCGGACCACuucgacaug

30   aacgcucaaGCGGCAACGGAGUCCUGA———ACGGACGGACCACCGCAAGAAu

2   cucaaGCGGACAGUAAACGACCCACGAUU—GCGAUGGGGACACAAGuucgacaug

3,23,29,32 cucaaAAGAAGGGUCAACGACCAACUCCU—ACAGUUGGGACACAACuucgacaug

10,24,36   caaUGAAUUGGAGUAAACGACUCACC————AAUGAGGACACAACCAAuucgac   II

34   gcucaaGGCGAUGGAAACGACGGAAAAGAGAAAAGUACGGACACAAuucgacauga

22   cucaaUCGAACGGUUAACGACCUCGAACA–CAAGGGGGGCACAAAAuucgacauga

15   aaGAGAAUCUAACGAGACGUAAGCCGUCGGAGAAUGAGACGAuucgacaugaggc

14,25,28   aGUGAGGACUAAUGAGGCGUACGA—CCGGAGAUUGAGACGUCuucgacaugagg   III
31,33

17   cgcucaaGGCGACGGGACUGCAAGCAUGGAGCUAACGAGAAAAUUGCuucgaca

1   cgcucaaCCUGGAGAGGAUCGCUGGCGGGCUUGAUCCCCAGAUCAAAuucgaca
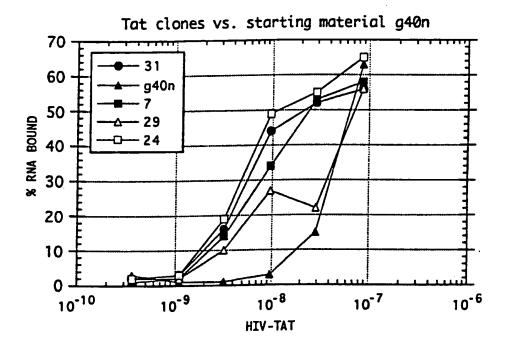
FIGURE 26

FIGURE 27

FIGURE 28

32/46

THROMBIN RNA BINDING SEQUENCES

CLASS I	1	2	3	SEQ I
#1	AGAUGCCUGUCGAGCAUGCUR	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#6	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#13	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#19	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#23	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#24	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#25	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137
#30	AGAUGCCUGUCGAGCAUGCUG	AGGAUCGAAGUUAGUAGGCUUUGUGUGCUC	GUAGCUAAACAGCUUUGUCGACGGG	137

#2 AGAUGCCUGUCGAGCAUGCUG UACUGGAUCGAAGUUAGUAGGCAGUCAC GUAGCUAAACAGCUUUGUCGACGGG   138

#5 AGAUGCCUGUCGAGCAUGCUG AUAUCACGGAUCGAAGGAAGUAGGCGUG GUAGCUAAACAGCUUUGUCGACGGG   139

#9 AGAUGCCUGUCGAGCAUGCUG CCUUUCCCGGGUUCGAAGUCAGUAGGCCGG GUAGCUAAACAGCUUUGUCGACGGG   140

#10 AGAUGCCUGUCGAGCAUGCUG CACCCGGAUCGAAGUUAGUAGGCGUGAGU GUAGCUAAACAGCUUUGUCGACGGG   141

#15 AGAUGCCUGUCGAGCAUGCUG UGUACGGAUCGAAGUAGUAGGCAGGUUAC GUAGCUAAACAGCUUUGUCGACGGG   142

#16 AGAUGCCUGUCGAGCAUGCUG CAUCCGGAUCGAAGUUAGUAGGCCGAGGUG GUAGCUAAACAGCUUUGUCGACGGG   143

#18 AGAUGCCUGUCGAGCAUGCUG AUUGUUGCGGAUCGAAGUGAGUAGGCGCUA GUAGCUAAACAGCUUUGUCGACGGG   144

#21 AGAUGCCUGUCGAGCAUGCUG UGUACUGGAUCGAAGGUAGUAGGCAGUCAC GUAGCUAAACAGCUUUGUCGACGGG   145

#22 AGAUGCCUGUCGAGCAUGCUG AUCGAAGUUAGUAGGAGCGUGUG GUAGCUAAACAGCUUUGUCGACGGG   146

#26 AGAUGCCUGUCGAGCAUGCUG ACGCUGGAGUCGGAUCGAAAGGUAAGUAGGCGACU GUAGCUAAACAGCUUUGUCGACGGG   147

#31 AGAUGCCUGUCGAGCAUGCUG GGGUCGGAUCGAAAGGUAAGUAGGCGACU GUAGCUAAACAGCUUUGUCGACGGG   148

#33 AGAUGCCUGUCGAGCAUGCUG AUAUCACGGAUCGAAAGAGAGUAGGCGU GUAGCUAAACAGCUUUGUCGACGGG   149

#34 AGAUGCCUGUCGAGCAUGCUG UGUACUGGAUCGAAGGUAGUAGGCAGGCAC GUAGCUAAACAGCUUUGUCGACGGG   150

#37 AGAUGCCUGUCGAGCAUGCUG AUAUCACGGAUCGAAGGAAAGUAGGCGUG GUAGCUAAACAGCUUUGUCGACGGG   151

CLASS II

#3	AGAUGCCUGUCGAGCAUGCUG	GUGCGGCUUUGGCGCCGUGCUUGGC	GUAGCUAAACAGCUUUGUCGACGGG	152
#20	AGAUGCCUGUCGAGCAUGCUG	GUGCGGCUUUGGGCGCCGUGCUUAC	GUAGCUAAACAGCUUUGUCGACGGG	153
#27	AGAUGCCUGUCGAGCAUGCUG	GUGCGGCUUUGGGCGCCGUGCUUGAC	GUAGCUAAACAGCUUUGUCGACGGG	154
#35	AGAUGCCUGUCGAGCAUGCUG	GGGCGGCUUUGGGCGCCGUGCUUGAC	GUAGCUAAACAGCUUUGUCGACGGG	155
#38	AGAUGCCUGUCGAGCAUGCUG	GUGCGGCUUUGGGCGCCGUGCUUGAC	GUAGCUAAACAGCUUUGUCGACGGG	154
#39	AGAUGCCUGUCGAGCAUGCUG	GUGCGGCUUUGGGCGCCGUGCUUGAC	GUAGCUAAACAGCUUUGUCGACGGG	154

FIGURE 29

CLONE	RANDOM REGION	SEQ ID NO:
6	gggaguugccuguc[g[agcaugcug AGGADCGAAGUUAGGUAGGCUUUCUGUGCU]C guagcuaaacagcuuuguucgacggg	156
16	gggagaugccuguucgugcau[gcug C[AU[CCGGAUCGAAGUUAGUAGGCCCAG]GUG guagcuaaacagcuuuguucgacggg	157
18	gggaguugccugucgagcaugcug AUUGU[UGCCGGADCGAAGUCGAGUAGGCGCUA] guagcuaaacagcuuuguucgacggg	158
27	gggagaugccuguc[g[agcaugcug GUGCGGGCUUUGGGGGCGCCCGDGCUU]GAC guagcuaaacagcuuuguucgacggg	159

FIGURE 30

POSSIBLE 2D STRUCTURES OF THROMBIN CLONES

FIGURE 30 CONT.

FIGURE 31

FIGURE 32

A.

Competition with DNA G15D Tracer

B.

Competition with RNA 33 Tracer

FIGURE 33
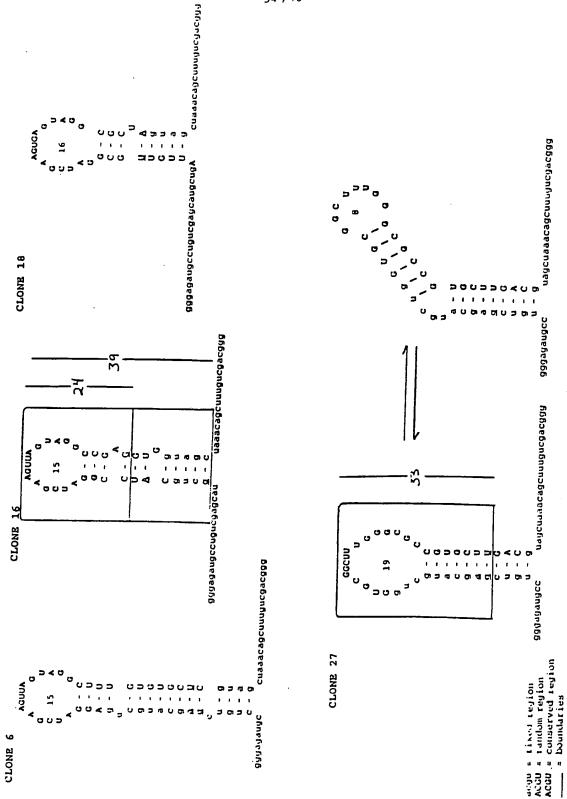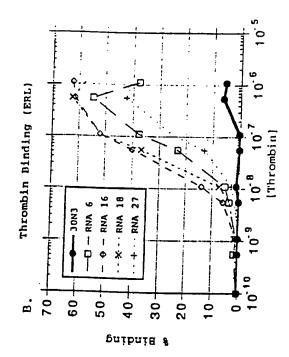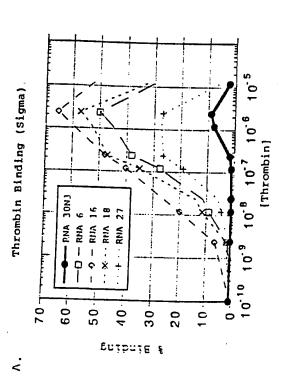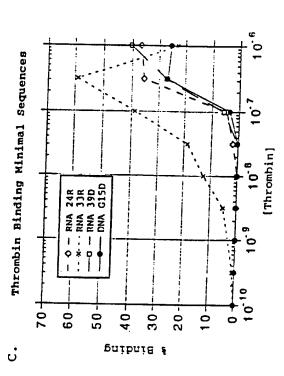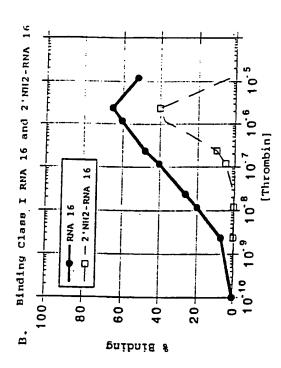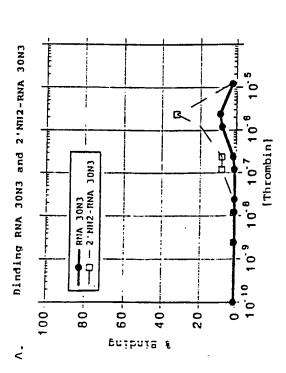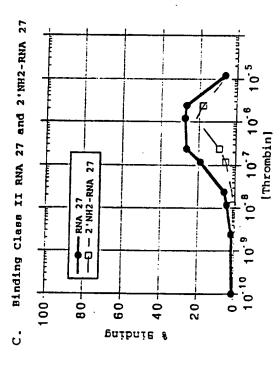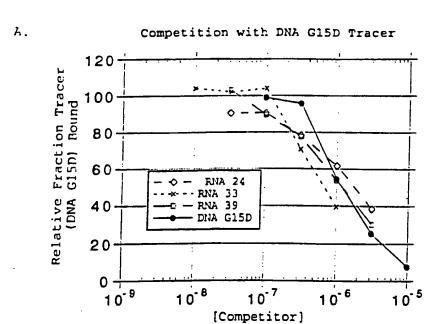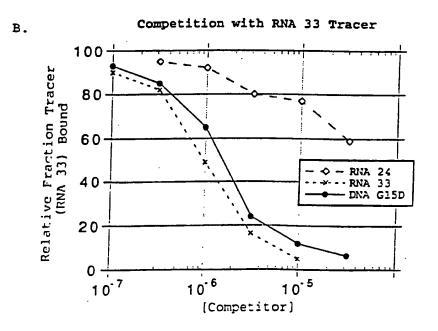
FUNCTIONAL ASSAYS THROMBIN ACTIVITY

A. Peptidase Activity-Cleavage of tripeptide p-nitroaniline substrate (S2238)

$$\text{H-D-Phe-Pip-Arg-p-Nitroaniline} + H_2O \xrightarrow{\text{Thrombin}} \text{H-D-Phe-Pip-Arg-OH} + \text{p-Nitroaniline}$$

Measure the OD 405 for release of p-Nitroaniline

	[Thrombin]	[RNA]	Inhibition (decrease in OD$_{405}$)
Class I RNA 16	10^{-8}M	10^{-8}M	—
	10^{-8}M	10^{-7}M	—
	10^{-9}M	10^{-8}M	—
Class II RNA 27	10^{-8}M	10^{-8}M	—
	10^{-8}M	10^{-7}M	—
	10^{-9}M	10^{-8}M	—
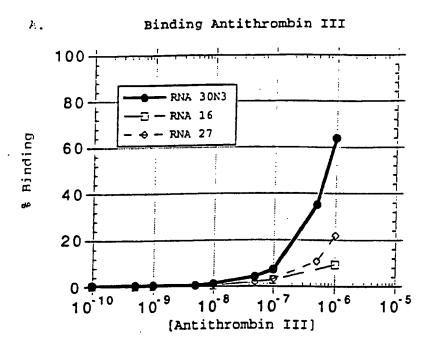
B. Fibrinogen Clotting Assay

Ligand plus purified human thrombin (2.5 nM)	Clotting time (sec) for purified fibrinogen (0.25 mg/ml)
No RNA/DNA	65
Class I RNA 16 (30nM)	117
Class II RNA 27 (60nM)	115
DNA 15mer G15D	270-330

FIGURE 34
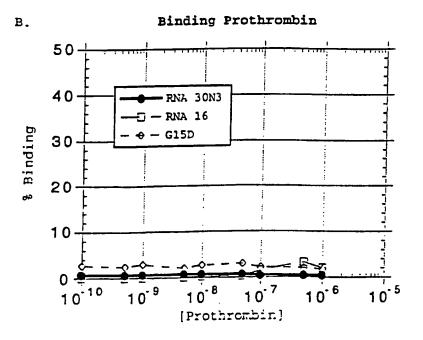
FIGURE 35

Figure 36

FIGURE 37

FIGURE   37b.

Figure 38

4A   gggagcucagaauaaacgcucaaUGCUAUUCGCCUAAACUCGGCGCUCCUACCUuucgacaugaggcccggauccggc

5A   gggagcucagaauaaacgcucaaAUCUCCUCCCGUCGAAGCUAACCUGGCACuucgacaugaggcccggauccggc

7A   gggagcucagaauaaacgcucaaUCGGCGAGCUAACCAAGACACUCGCUCCuucgacaugaggcccggauccggc

10A   gggagcucagaauaaacgcucaaGUAGCACUAUCGGCCUAAUCGGCCUuucgacaugaggcccggauccggc

13A   gggagcucagaauaaacgcucaaACCCGCGGCUCCGAAGCUAACCAGGACACuucgacaugaggcccggauccggc

14A   gggagcucagaauaacgcucaaUGGGUGCUAACCACGCACGACACUGUuucgacaugaggcccggauccggc

16A   gggagcucagaauaaacgcucaaCACGCACGCUAACCAGCUAAGCCACUGUCCCuucgacaugaggcccggauccggc

18A   gggagcucagaauaaacgcucaaCUGCGUAUAACCACUGUAucgacaugaggcccggauccggc

21A   gggagcucagaauaaacgcucaaUGGGUGCUUAAACCAGUCGCACACCUGCUGuucgacaugaggcccggauccggc

25A   gggagcucagaauaaacgcucaaCUAGGUGCUAUCCAGACUCUCCUuucgacaugaggcccggauccggc

29A   gggagcucagaauaaacgcucaaUGCUAUUCGCCGGCUCCGGCUCCUACCUuucgacaugaggcccggauccggc

38A   gggagcucagaauaaacgcucaaAGCUAUUCGCCUGCGUCCCCAACCCGCAACGCACAUGCCCuucgacaugaggcccggauccggc

39A   gggagcucagaauaaacgcucaaAGCUGCGUCAACGCACAUGCCGuucgacaugaggcccggauccggc

56A   gggagcucagaauaaacgcucaaCAGGCCCCGUCGUAAGCUAACCUGGCCGuucgacaugaggcccggauccggc

FIGURE 39

FIGURE 40

A   C

A   C

$(Y)_{0-1}$ $(N)_{0-2}$

Y - Y'

$(N - N')_{1-4}$

$(N)_{0-4}$ $(N)_{2-5}$

$(N - N')_{0-6}$

FAMILY 1

N   N   G
W   D
Y   C
G   A
C   A
A   G
A   N
H   N
G   A   C
G - C
R - Y
R - Y
N - N'

FAMILY 2

FIGURE 41

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34; C07H 21/02, C07H 21/04

US CL :435/6, 435/91.1; 536/22.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 435/91.1; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE, Vol. 249, issued 03 August 1990, Craig Tuerk and	1, 6, 9, 10, 33
Y	Larry Gold, "Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase", pages 505-510. See entire document.	2-5, 7, 8, 11-32, 34-83
X	NATURE, Vol. 355, issued 06 February 1992, Louis C. Bock et	1, 6, 9, 10, 33
Y	al., "Selection of Single- Stranded DNA Molecules That Bind and Inhibit Human Thrombin", pages 564-566. See abstract and Figure 1 at page 565.	2-5, 7, 8, 11-32, 34-83

| X | Further documents are listed in the continuation of Box C. | ☐ See patent family annex. |

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 DECEMBER 1993	12 JAN 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	STEPHANIE W. ZITOMER, PHD.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	NUCLEIC ACIDS RESEARCH, Vol. 18, No. 11, issued 1990, Hans-Jurgen Thiesen and Christian Bach, "Target Detection Assay (TDA): a Versatile Procedure to Determine DNA Binding Sites as Demonstrated on SP1", pages 3203-3209. See abstract and Figure 1 at page 3204.	1, 6, 9, 10, 33 2-5, 7, 8, 11-32, 34-83